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# *In vitro* enhancement of retention and vascular support capacity of bone marrow derived stem cells for cardiac repair

Thesis submitted for the degree of

Doctor of Engineering in

Biochemical Engineering

**Declaration**

I, Owen William Bain confirm that the work presented in this thesis is my own.

Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

## Acknowledgements

No great achievement is completed alone, this thesis is no different. From my fellow colleagues to the inspiration of words from journal articles of scientists I've never met. It was an incredibly humbling journey to the end.

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## Abstract

Cardiovascular disease is a worldwide problem as the number one cause of death and disease. Although improvements in pharmaceutical and surgical interventions have increased patient survival post infarction, approximately half those that recover have a progress to congestive heart failure.

Stem cell therapy is a strategy to improve functional recovery after myocardial injury. Many early phase clinical trials have been conducted throughout the world, albeit with variable results. It is postulated that the transplanted cells are not retained within the peri-infarct region and either apoptose or migrate to the spleen.

This thesis aims to understand the functional characteristics of bone marrow mononuclear cells (BM-MNC) and Mesenchymal Stem Cells to characterise their adhesion and migration responses that are essential for successful cell retention.

*In vitro* model assays were developed to mimic the physiological environment of the peri-infarct region using hypoxic conditions and fibronectin, an extracellular matrix that is highly upregulated in the infarct region.

*In vivo* rat studies have shown Stromal Derived Factor-1 (SDF-1) stimulated MSCs improved cardiac function post infarction. In this study SDF-1 stimulation had no effect on the *in-vitro* adhesion and chemotaxis of MSCs and BM-MNCs. Both cell types had significantly decreased adhesion to fibronectin in hypoxic conditions

To investigate the importance of Neuropilin-1 (NRP1) MSCs were knockdown for the protein. NRP1 knocked down MSCs had significantly decreased

adhesion to fibronectin, chemotaxis to PDGF-AA, and interactions with endothelial cells. Expression of NRP1 was enhanced by basic fibroblast growth factor that enhanced adhesion in hypoxic conditions and increased vascular endothelial growth factor release.

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**List of abbreviations**

| | |
|---|---|
| 5AZT | 5 - azidothymidine |
| AMI | Acute myocardial infarction |
| aSMA | alpha smooth muscle actin |
| bFGF | basic fibroblast growth factor |
| BM | bone marrow |
| BMP2 | Bone morphogenic protein 2 |
| BMSC | bone marrow stem cell |
| BSA | bovine serum albumin |
| CHD | coronary heart disease |
| CM | cardiomyocyte |
| CSC | cardiac sateilite cell |
| CVD | coronary vascular disease |
| Cx-43 | Connexion 43 |
| CXCR4 | C-X-C chemokine receptor type 4 |
| DAPI | 4,6-diamidino-2-phenylindole |
| DCM | Dilated cardiomyopathy |
| DMEM | Dulbecco's modified eagle's medium |
| DMSO | Dimethyl sulfoxide |
| ECC | Epithelial endometrial cells |
| ECM | Extracellular matrix |
| EDTA | Ethylendiaminetetetracetic acid |
| EGM-2 | Endothelial growth medium-2 |
| EPC | Endothelial progenitor cell |
| FBS | Fetal bovine serum |
| FGF-2 | Fibroblast growth factor 2 |
| FN | Fibronectin |
| GvHD | Graft versus host disease |

| | |
|---|---|
| HGF | Hepatocyte growth factor |
| HIF-1 | Hypoxia inducible factor 1 |
| HSC | Haematopoetic stem cell |
| HSP70 | Heat shock protein 70 |
| HUVEC | Human umblical cord vein endothelial cell |
| icam-1 | Intercellular Adhesion Molecule 1 |
| IGF-1 | Insulin growth factor 1 |
| IgG | Immunoglobulin G |
| IHD | Ischemic heart disease |
| il-13 | Interleukin 13 |
| il-4 | Interleukin 4 |
| KD | Knockdown |
| LDL | Low density lipoprotein |
| LPS | Lipopolysaccaride |
| LVEF | Left ventricular ejection factor |
| MCP-1 | Monocyte chemoattractant protein |
| M-CSF | Macrophage colony stimulating factor |
| MMP | Matrix metalloproteinases |
| MNC | Mononuclear cell |
| MSC | Mesenchymal stromal cell |
| NF-kB | Nuclear factor - kappa B |
| NRP1 | Neuropilin 1 |
| PBS | Phosphate buffered saline |
| PCI | Percutaneous coronary intervention |
| PDGF-aa | Platlet derived growth factor aa |
| PECAM | Platelet endothelial cell adhesion molecule |
| PFA | Paraformaldehyde |
| PI3K | Phosphatidylinositol 3-kinase |

| | |
|---|---|
| PVDF | Polyvinylidene difluoride |
| qPCR | quantified polymerase chain reaction |
| SCF | Stem cell factor |
| SDF-1 | Stromal derived factor 1 |
| SDS-PAGE | Sodium dodecyl sulphate - polyacrilamide gel electrophrosesis |
| sFRP2 | secreted frizzled related protein |
| siRNA | small interfering ribonucleic acid |
| T/E | Trypsin/EDTA |
| TNF-a | Tumour necrosis factor alpha |
| VCAM-1 | Vascular cell adhesion protein 1 |
| VEGF | Vascular endothelial growth factor |
| VEGFR | Vascular endothelial growth factor receptor |
| VLA-4 | Very late antigen-4 |

# Chapter 1 – Introduction

Cardiovascular disease (CVD) is the number one cause of mortality and morbidity in the world (WHO, 2005). CVD is a huge burden on the UK economy, costing over £30.7 billion a year (Allender, 2008). If treatment is not improved it is predicted by 2030, almost 24 million people will die per year from CVDs worldwide, with heart disease being the most predominant form of CVD. In the UK alone approximately 150,000 people a year have a myocardial infarction (MI; Heart attack)(BHF, 2008). In the past half century, mortality rates from acute myocardial infarction (AMI) have decreased due to advances and breakthroughs in pharmacological therapies and surgical intervention, leading to >90% survival rate 30 days post-MI (White et al., 2005). However, injured myocardium is never fully repaired and the patient is likely to develop congestive heart failure, the leading cause of mortality in post-MI patients that have increased in number as more survive. This is because after an AMI the ischemic region of the heart becomes necrotic and non-contractile collagenous scar tissue is deposited, so the heart cannot pump as efficiently. With a region of the heart non-viable, the surrounding myocardium becomes overburdened, and so the cardiac function deteriorates.

Adult stem cells represent a new potential therapeutic in cardiovascular medicine. Developments in stem cell biology have resulted in their identification as potential candidates for the treatment of heart disease. Numerous studies, in animal and human, have used cell populations loosely termed bone marrow stem cells (BMSC) for chronic heart failure and acute MI indicating a significant therapeutic benefit. Despite these studies the

precise mechanism of action remains elusive. Conflicting reports suggest models of either paracrine, immunomodulatory, or regenerative paradigm.

BMSCs are a very broad range of cells including endothelial progenitor, mesenchymal multipotent adult progentior and haemopoeitc stem cells. Although there have been numerous clinical trials evaluating their collective potential, few trials have used individual populations. However, there have been numerous animal studies selectively separating the populations for cardiac repair.

This review will concentrate on the use of bone marrow stem cells in AMI, while acknowledging their use in chronic heart disease. It will focus on the recruitment of BMSCs to the infarct region, and discuss the potential mechanism of the derived benefits. By utilising this knowledge there is great scope for translating sound basic science to improve the modest effects of BMSC achieved in clinical trials which one day may become a recognized therapy.

# 1. The Heart and Myocardial Infarction

## 1.1 The Heart

The heart is a muscular organ, composed of cardiac muscle tissue that pumps blood to the lungs and the rest of the body. The heart consists of 4 main chambers, 2 atrium and 2 ventricles. The atria receive blood coming into the heart and deliver blood to the ventricles. The right atrium receives deoxygenated blood and delivers it to the right ventricle to be pumped to the

lungs. The left side receives oxygenated blood and pumps it to the rest of the body. Therefore the left ventricle muscle wall is much thicker than that of the right ventricle. While the heart delivers blood to the rest of the body, it is supplied with oxygenated blood via the coronary arteries that ensure adequate delivery to the myocardium. The 3 main coronary arteries are 2 on the left and  one on the right that originate from the aorta (Katz, 2005).

The heart wall consists of the epicardium, the membrane that covers the heart, the thick muscular layer of the myocardium, and the endocardium, the inner lining. These layers are contained within a double walled sac called the pericardium (Figure 1).

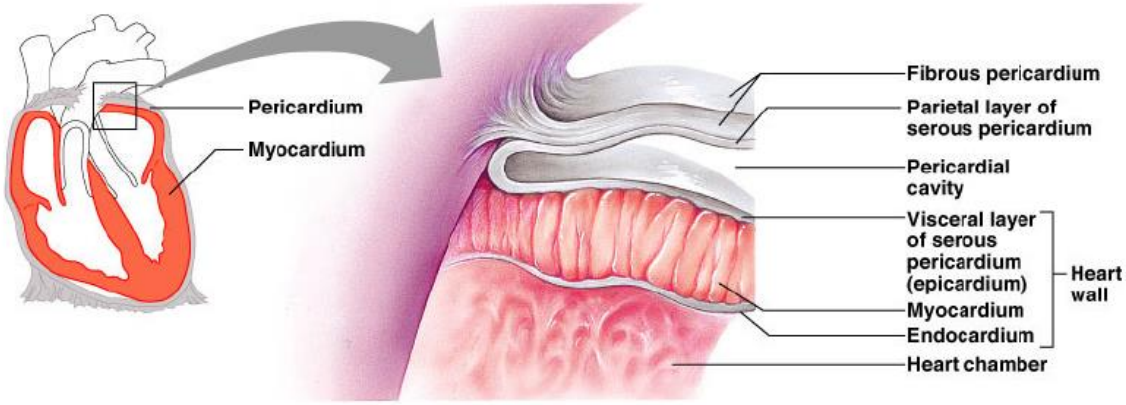



Figure 1 - Anatomy of the heart (Carter, 2012)

The myocardium comprises predominantly of cardiomyocytes that pump the blood round the body. The myocardium is enveloped by the endocardium and the epicardium

Contraction of the muscle cells, cardiomyocytes (CM), is synchronised to ensure coordinated ventricular contraction. In order to do this the cardiomyocytes are interconnected by contiguous cytoplasmic bridges.

Contraction of the CM is generated by an action potential in the pacemaker cells in the sino-atrial node. This is transmitted throughout the atria through the A-V node, to initiate a contraction. Simultaneously, this transmission causes the excitation of the atrio-ventricular node that spreads the excitation to the ventricles causing a coordinated ventricular contraction. This mechanism is repeated allowing a rhythmic wave like pumping action allowing blood to be received in and efficiently pumped out (Katz, 2005).

Although, myocytes account for over 90% of the myocardium volume, due to their comparative large size they only comprise of a quarter of the cells in the heart. The rest consists of fibroblasts and endothelial cells making up the dense capillary network. The cardiomyocytes consist of the cell membrane, sarcolemma, and T tubules for impulse conduction, and the sarcoplasmic reticulum, a calcium reservoir for contraction. The contractile unit is an arrangement of thick filaments composed of myosin and thin filaments containing actin that makes up the sarcomere. Sarcomeres also contain troponin and tropomyosin. The usual striated appearance of CMs is due to arrays of sarcomeres in series (Figure 2). This arrangement allows contraction of the cardiac muscle by sliding of the actin filaments between the myosin, toward the centre of each sarcomere. Compared to skeletal muscle cells, cardiac muscle contains a substantial amount of mitochondria emphasising consistently high metabolic requirement. Therefore, if a region of myocardium is deprived of oxygen for even a short period, severe consequences that can ensue, leading to loss of cardiac function or death.

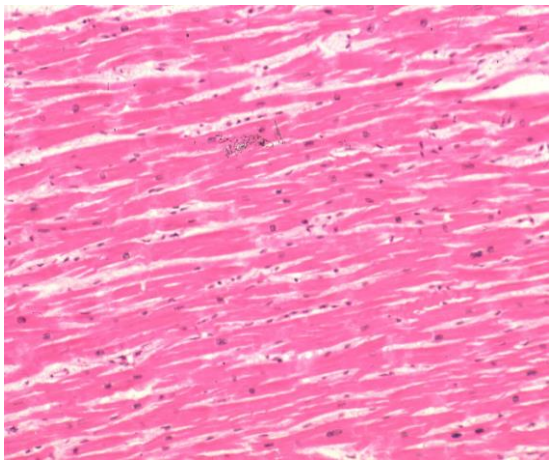


Figure 2: Striated appearance of cardiomyocytes in the myocardium(Skyschally et al., 2008)

Myocardial Infarction commonly occurs in patients with Coronary Heart Disease (CHD), when sudden rupture of a pre-existing atherosclerotic plaque occludes a coronary artery. There is often no warning and for approximately 50% of CHD cases, myocardial infarction is the first manifestation of the disease. The underlying pathophysiology of CHD is accumulation of atherosclerotic plaques within the walls of the coronary arteries. The formation of this plaque can involve inflammation, often initiated by dyslipidemia or hypertension that triggers enhanced monocyte recruitment into the arterial wall (Jefferson and Topol, 2005). Upon attachment to the endothelium these monocytes differentiate into macrophages expressing surface scavenger receptors. The receptors enable the macrophages to uptake oxidised low density lipoprotein (LDL) by endocytosis. Eventually foam cells are formed, and cholesterol is deposited in the plaque. When the foam cells die their constituents are released attracting more macrophages increasing the plaque burden. The genesis of MI is caused by the fissure of the maturing plaque, allowing the

constituents to exude into circulation, and an acute thrombus is formed (Jefferson et al 2005). This rupture leads to the occlusion of a coronary artery, blocking bloody supply, rendering the affected area of the heart ischemic. (Figure 3)

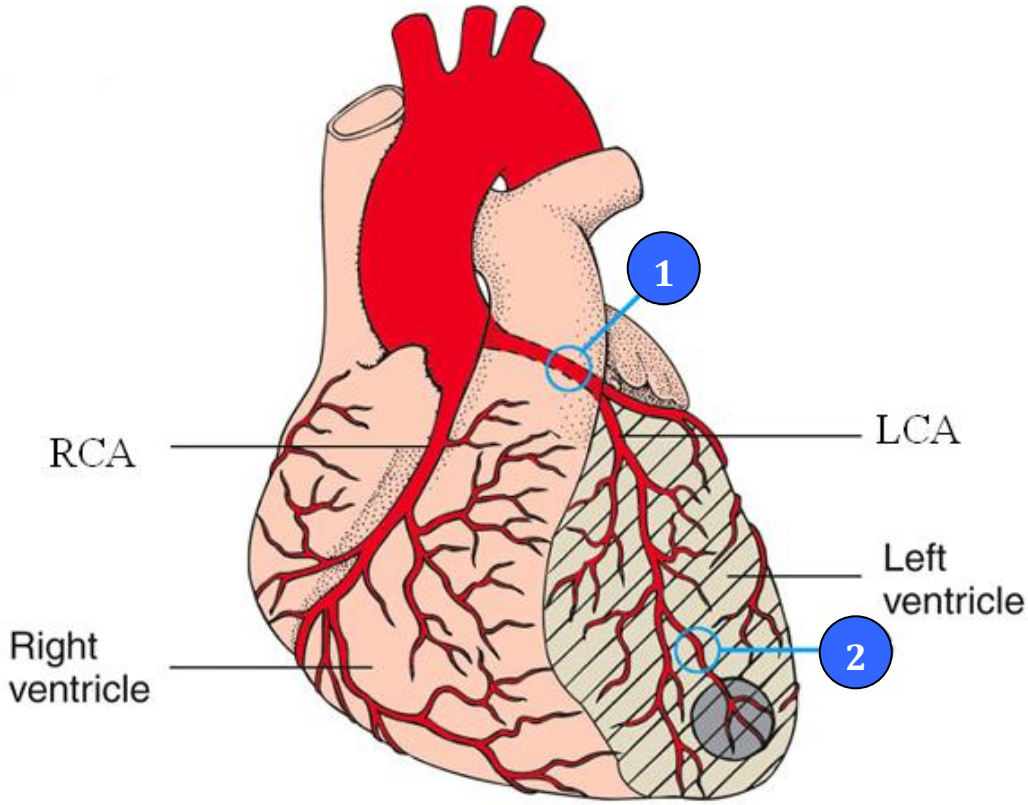



Figure 3 - Occlusion of a coronary artery is the cause of myocardial infarction

1 - occlusion of left coronary artery due to ruptured athersclerotic plaque, large area left ischemic ; 2 - An occlusion lower down, less myocardium is affected-; LCA – Left coronary artery; RCA – Right coronary artery (Jefferson et al 2005)

When cardiac tissue is deprived of oxygen the myocardium becomes hypoxic triggering an ischemic cascade causing necrosis. Necrosis spreads from the centre to the border of the occluded vascular region

(Skyschally et al., 2008). The final result is a non-contractile collagen scar. The size of the infarct region depends on several factors including residual blood flow through the collaterals during ischemia, heart rate and the location of the perfusion area distal to the occlusion. The final infarct size develops over several hours. Therefore, as long as the collateral blood flow is sufficient, some ischemic myocardium can potentially be rescued during reperfusion (Schaper and Schaper, 1988). Coronary collateral blood flow is vital for response to acute and chronic ischemia(Hakimzadeh et al., 2014). Collateral vessels result from arteriogenesis of pre-exisiting intercoronary anastomoses (Heil and Schaper, 2004). Arteriogenesis is promoted by the fluid shear stress detected by the vascular endothelium that causes the enlargement of collateral vessels through growth and proliferation. The endothelium releases several chemokines that attract monocytes that mature to macrophages. The macrophages secrete other growth factors and cytokines such as basic fibroblast growth factor and TNFα aswell as matrix metalloproteinases that break down the surrounding extracellular matrix allowing space for the growing vessel (Fung and Helisch, 2012). These vessels can grow to the extent that they take over the role of the occluded artery (van Oostrom et al., 2008). In patients with adequate collateral flow, an acute coronary occlusion ischemia may not occur.

In rodents, the time scales are different, with the final infarct size in rodents with high heart rate and low collateral blood flow being reached after 30 minutes (Verdouw et al., 1998). This is important to factor in when assessing data from mouse and rat MI models

### 1.1.1 Inflammatory response

Inflammation, in addition to being a contributing factor to the initiaton of MI, has a major impact post-MI. During myocardial ischemia mitochondria from the cardiomyocytes are extruded through breaks in the sarcolemma and unfold, releasing membrane fragments rich in cardiolipin and protein. These fragments bind to C1 triggering the complement inflammatory cascade (Frangogiannis, 2008). Complement activation is followed by neutrophil and monocyte recruitment to the infarct area (Rossen et al., 1994),

After inflammatory induction, neutrophils extravasate into extravascular tissue in response to chemotactic stimuli (Figure 4). Those cardiomyocytes expressing ICAM-1 bind neutrophils, via the Mac-1(CD11b/CD18)/ICAM-1(CD54) interaction. Expression of ICAM-1 is known to be upregulated after MI following complement binding (Niessen et al., 1999). This adhesion triggers the neutrophil respiratory burst that is cytotoxic to the adhered cell. The extent of myocardial injury is neutrophil dependant as neutrophils become entrapped in the microvasculature preventing reperfusion. It has been demonstrated that depletion of neutrophils in animal models undergoing reperfused myocardial infarction led to a decrease in infarct size (Kin et al., 2006).

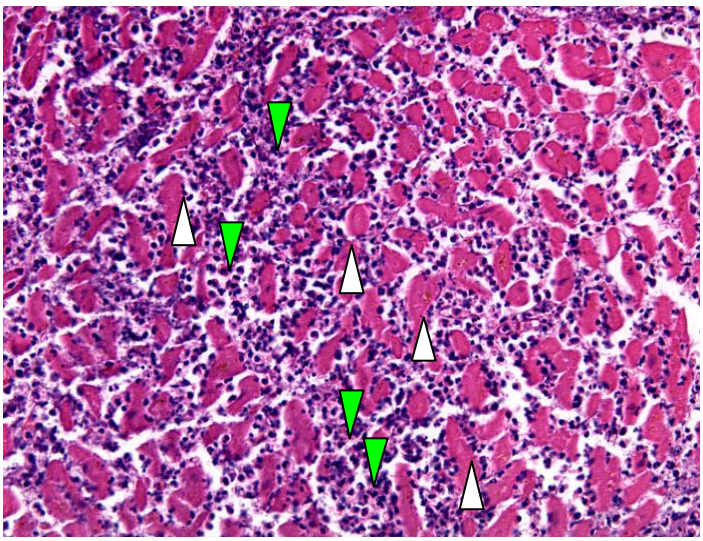


Figure 4 Invasion of ischemic myocardium by neutrophils (Kin et al., 2006).

White arrows - cardiomyocytes; Green - neutrophils

Macrophages are heavily involved post-MI, having an active role in inflammation, scar formation and scar remodelling (Lambert et al., 2008). Signals from resident cells and neutrophils account for the monocyte migration towards the injured myocardium, especially the chemokines monocyte chemoattractant protein (MCP-1;CCL2) and TNF-α. This infiltration occurs predominantly after reperfusion. Monocytes are postulated to be recruited from the spleen as post-mortum analysis has shown accumulation of monocytes in the myocardium correlating with depletion in the spleen (van der Laan et al., 2014).

Once the monocytes adhere to the ECM the macrophage conversion is triggered (Lambert et al., 2008). The activation of macrophages is divided into two groups: proinflammatory (M1) and anti-inflammatory (M2). M2 macrophages promote angiogenesis and ECM reconstruction, whereas M1 macrophages initiate ECM destruction after induction by lipopolysaccaride. After MI macrophages are activated initially via the M1 pathway and then by the M2 pathway. The M2 macrophages are induced by glucocorticoids and ingest apoptotic neutrophils and necrotic myocytes, facilitating the wound healing process (Luikart et al., 2006). These M2 macrophages are derived from mature M1 macrophages by treatment with interleukin 4 (IL-4) or IL -13 (Ho and Sly, 2009). M2 macrophages release TGFβ1 initiating collagen release from the fibroblasts in the ischemic myocardium. This inflammatory response improves tissue repair, and several studies have demonstrated that stimulating macrophages with macrophage- colony stimulating factor (M-CSF) results in improved cardiac function (Okazaki et al., 2007). Furthermore, Yano et al showed that macrophage depletion leads to impaired wound healing, highlighting the importance of the macrophage (Yano et al., 2006).

In addition to monocytes, CD34+ mononuclear cells are mobilised in response to myocardial infarction although their role in myocardial regeneration has yet to be established (Theiss et al., 2007)

Additional inflammatory responses are regulated by the complex formation of nuclear factor kappa beta (NF-κβ) and Inhibitor of κβ (Iκβ). NF- κβ is a protein complex that regulates the transcription of many genes involved in proliferation and cell survival particularly in macrophages. Activation of NF-

κβ is mediated by the release of cytokines and free radicals that are predominantly released by mast cells. TNFα is a cytokine implicated in the initiation of this cytokine cascade. By knocking down both TNF 1 and 2, receptors causes larger infarcts are evident after MI, and increased myocyte apoptosis is observed compared to controls (Kurrelmeyer et al., 2000).

### 1.1.2 Endogenous stem cell repair

The heart was long assumed to be post-mitotic containing purely terminally differentiated cells rendering the post-natal heart unable to regenerate after damaged. However, recent evidence has identified an endogenous reserve of cardiac stem cells (CSCs) and early committed cells (ECCs) (Urbanek et al., 2005). These cells are referred to as cardiac side populations (CSP) and are found in both the atria and ventricles. They are able to give rise most cell types in the heart including cardiomyocytes, fibroblasts, endothelial cells and smooth muscle cells (Kajstura et al., 2008). Under normal conditions, cardiomyocytes and fibroblasts have a very low turnover, so only a small number of CSPs are required. This is contrary to tissues and organs such as the gut and oral mucosa that both have a higher proportion of stem cells due to high cell turnover. CSPs are classified into subtypes based on their surface marker characterisation eg. Sca-1+ and c-kit+, while ATP binding cassette subfamily group 2 (Abcg2), a has been identified as a marker for all these CSPs (Meissner et al., 2006). These cells have been identified in the ischemic border zone following MI, indicating a cellular response to cardiac injury by these populations but it is not significant to fully repair the heart after MI. The reduced vascular perfusion to the infarct area may reduce the

activation of resident CSP cells. However, in vivo studies have shown that injection of CSPs into infarcted myocardium increases ventricular function and regenerates necrotic tissue (Kajstura et al., 2008).

## 1.2 Burden of myocardial infarction

After the inflammatory phase granulation tissue is established, and cardiac fibroblasts proliferate. These cells migrate out of the granulation region leaving a matrix rich in collagen. The apoptotic cells lead to expression of anti-inflammatory cytokines and TGFβ, which initiates the transition from inflammation to fibrosis (Frantz et al., 2009). Ventricular remodelling occurs that leads to the expansion of the initial infarct area and fibrous tissue replacing cardiomyocytes in the ventricular wall (Kocher et al., 2001). The collagen scar leaves a burden on remaining viable cardiomyocytes in the surrounding margins, leaving them over-stretched. After an AMI, if the patient survives, the heart is permanently damaged. They are now susceptible to further cardiac events such as ventricular fibrillation and ventricular tachycardia. The scar region impairs normal cardiac function, and the infarct area could extend as cardiac output and blood pressure fall leading to further coronary ischemia. The tightly woven collagen has high tensile strength and prevents contraction, hence reducing contractile ability and cardiac output. The cardiomyocytes surrounding the infarct region are continually lost after the MI, increasing the risk of heart failure.

### 1.3 Current treatment

Current pharmaceutical interventions aim to reduce burden on the heart by regulating parameters such as blood pressure and heart rate. However, they are not regenerate and do not aid in restoration of damaged tissue or stimulation of dormant cardiomyocytes. Immediately after MI, aspirin and an anti thrombyltic, such alteplase, are administered to prevent clot build up and enhance reperfusion to the ischemic region (Chua et al., 2005). Other acute interventions include percutaneous coronary intervention (PCI), a non-surgical intervention that involves widening the coronary artery via the insertion of a balloon catheter to dilate the artery from within. A metallic stent can then be inserted once the artery has been dilated (Smith et al., 2006).

Following MI the patient is given multiple medicines, including aspirin, an angiotensin II converting enzyme inhibitor, a statin (3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor) and a beta antagonist.

BMSCs have the potential to overcome, or at least alleviate, this long term requirement for medication. Although clinical trials have been conducted using BMSCs, clarification of the mechanism of action is required to optimise the therapeutic response.

## 2. Bone Marrow Stem Cell trials for cardiac repair

Stem cell populations, by definition, can undergo both self –renewal and differentiation into at least one functional cell population. Observations that bone marrow derived cells can differentiate along the myogenic lineage have

promoted researchers to investigate the potential benefits of these cells following MI. Promising results from animal studies have led to the initiation of phase I and II trials for use of BMSCs in human. So far, numerous trials have been performed and published on the transplantation of BMSC. Orlic and colleagues pioneered the first trial in mouse showing that cells from the bone marrow could regenerate infarct myocardium (Orlic et al., 2001). However, despite initial promise, subsequent trials have failed to significantly build on this, and benefits reported in animal studies have not yet been fully translated to human benefit as there have been few phase III trials. Current data from trials suggests that patients most likely to derive benefit from bone marrow stem cells are those with large infarct and substantially reduced left ventricular ejection fraction (LVEF) (Drexler and Wollert, 2009). LVEF is commonly used as an immediate measure of clinical outcome and represents the fraction of blood pumped out of the left ventricle, calculated by the difference between end-diastolic and end-systolic volumes, and the stroke volume (SV), divided:

$$E_f = \frac{SV}{EDV} = \frac{EDV - ESV}{EDV}$$

Most of the following trials used autologous bone marrow cells. By using autologous cells, the potential of rejection is bypassed, as allogeneic cells risk graft versus host disease (GvHD) for which the patient would have to be immunosuppressed (Wolf et al., 2009). However, as a longer term aim, allogenic cells are likely more desirable due to the potential “off the shelf” availability they promise

### 2.1.1 Clinical trials

Several phase I and II trials have investigated the effects of BMSC for cardiac repair, with LVEF the most common endpoint. The TOPCARE-AMI trial was one of the first randomised clinical trials using progenitor cells in AMI (Schachinger et al., 2004). This trial compared the use of autologous BMSC against circulating blood progenitor cells administered 5 days after percutaneous coronary intervention (PCI). Both groups had an increased in LVEF and increased myocardial viability in the infarct zone, however there was not an appropriate control group to compare this to. There were no significant differences between the two cell populations used. However, the trial did highlight the potential of progenitor cell therapy in AMI with sustained improvement in cardiac function over 5 years (Yousef et al., 2009).

In the BOOST study, although LVEF was significantly improved by 6% compared to controls after 6 months (the primary end point), this was not sustained over 18 months. Although again appropriate placebo controls were not used. The trial investigated use of autologous BMSC in patients 5 days after MI. The increased LVEF after 6 months was associated with improvement in systolic wall motion (Wollert et al., 2004). After 18 months initial improvements in LVEF were not sustained.

The REPAIR-AMI trial investigated the infusion of basement membrane derived mononuclear cells 4 days after PCI. Their results indicated slight improvement in LVEF after 4 months that was sustained over 12 months, with mortality benefit observed (Marenzi and Bartorelli, 2007). These trials used a heterogenous population of cells, so the precise subset of clinically

advantageous cells is not known. The REGENT trial, by Tendera and colleagues, addressed this issue by investigating CD34+/CXCR4+ mononuclear BMC comparing to to unfractionated mononuclear BMC as a reference point (Tendera et al., 2009). However, no significant difference was observed between the groups, which both showed an improvement of ~3% in LVEF. Despite these results, many trials have shown this therapy to be safe.

The MAGIC Cell trial compared the administration of with Granulocyte – colony stimulating factor (G-CSF) alone versus G-CSF and BMSC. They found that the patients treated with G-CSF and BMSC had significantly greater improvement in LVEF after 6 months than G-CSF alone(Kang et al., 2007). This indicates that mobilisation of endogenous stem cells by G-CSF alone is not adequate enough to have a therapeutic consequence. G-CSF is regularly use in clinical practise for bone marrow transplantation, as it stimulates the bone marrow to produce granulocytes and stem cells to be released into the blood. The follow up to MAGIC Cell-3-DES trial involved a larger amount of patients, and reported improvements in LVEF were reported after patients with AMI were administered G-CSF followed by intracoronary injection of mobilised BMSCs. Another larger trial of 50 patients reported the similar improvements in LVEF after 6 months following after injection of BMSC mobilised by G-CSF (Steinwender et al., 2006).

The Cochrane review for ‘Stem cell treatment for acute myocardial infarction’ produced the most comprehensive analysis of randomised controlled trials to date (Martin-Rendon et al., 2008). Their main results, from 1765 patients, showed a large amount of heterogeneity between studies but overall

moderate improvements in heart functions were significant both short and long term (Martin-Rendon et al., 2008).

The BALANCE study, treating 62 patients with BMSCs after AMI and 62 control. This included a long term follow up for all patients after 5 years. Results showed an increase in contractility in the infarcted zone (Yousef et al., 2009). Most importantly, mortality was decreased and significantly augmented exercise capacity compared to the control group.

However, use of BMSCs for ischemic heart disease (IHD) has produced indifferent results. The FOCUS-CCTRN trial demonstrated no significant change in left ventricular end systolic volume or clinical improvement compared to placebo groups. This is in contrast to several meta-analyses that identify transplantation as favourable for IHD (Clifford et al., 2013). It is worth noting that non-publication of negative results hinders a true comparison (Ioannidis (Ioannidis, 2005)

Most studies have failed to detect BMCs in the myocardium 3 weeks after infusion, despite the maintenance of hemodynamic and anatomical improvement (Freyman et al., 2006b; Hale et al., 2008). Approximately 90% of transplanted cells die shortly after transplantation as a result of physical stress. A consensus on the optimal delivery method and timing of administration is desperately needed.

Use of mesenchymal stromal cells have only been used in a few published clinical trials, the majority of which are safety studies. The POSEIDON trial investigated the use of allogenic and autologous bone marrow derived MSCs in patients with ischemic cardiomyopathy Results from this study

demonstrate an encouraging safety profile, with improvements in quality of life, and NYHA classification indicated functional improvement (Hare et al., 2012). However, the results of this trial are limited as there was no placebo arm included and there were only 5 patients in each experimental group.

The safety study for Prochymal, the Osiris MSC product, was shown to be safe in 53 patients after AMI via intravenous infusion. After 6 months LVEF and pulmonary function were significantly improved in hMSC treated versus placebo treated.

Yang et al chose a different transplantation approach, with delivery via a non-infarct artery to repair AMI. Results after 6 months showed increased cardiac function and myocardial infusion. While no major incidences of adverse events following transplantation of MSCs for cardiac repair have been reported

### 2.1.2 Trial limitations

These clinical trials are incomparable as relevant variables, such as method of cell delivery, site of delivery, primary outcome, condition treated and timing of administration, are rarely consistent from study to study. Variability between different cohorts of patients can affect the end outcome too (Martin-Rendon et al., 2008). For instance delivery of cells via intravenous injection can be especially problematic in patients with poor circulation. Therefore, even before taking into account any other variable, results obtained will be largely affected.

Unlike animal studies it is difficult to track cellular differentiation after transplantation. Therefore, the mechanism of action remains elusive, and is

likely to be multi-factorial considering the distinct cell lineages transplanted. Characterisation of these cells presents a problem, as even cells from the same lineage can have varying cell surface marker expression depending on their maturity (George, 2010). Most studies have correlated CD34+ and CD133+ expression with angiogenesis and improved cardiac function. Derivation of specific cell types depending on their functionality is required. Regardless of the cell populations used, the optimal number of cells delivered is unclear. Nearly all clinical trials use a different amount ranging from 1 $x10^7$ to 5 x $10^9$, a 500 fold difference (Bartunek et al., 2007; Tatsumi et al., 2007).

Additionally, LVEF might not be an appropriate end point as it can often underestimate the therapeutic benefit to patients. Even very modest increases in LVEF can result in long term positive clinical outcomes. This has been addressed by the ASTAMI trial that accessed exercise time over a short and long period of time after transplantation to LVEF evaluation (Beitnes et al., 2009). They established significant improvements in BMSC patients. Another Phase II trial currently underway, REGENERATE-AMI, will access the patients Quality of Life 6 months and 1 year after the procedure (Hamshere et al., 2014; Yeo and Mathur, 2009).

## 3. Bone Marrow cell populations for myocardial repair

Although progenitor cells are recruited from the bone marrow to site of cardiac injury, endogenous reserves do not provide a critical mass capable of repair after MI. The bone marrow contains a variety progenitor and endothelial subsets. Subpopulations that may have the potential to be used

to repair injured myocardium include: endothelial progenitor cells (EPC), mesenchymal stromal cells (MSC), and haematpoetic stem cells (HSC). Although clinical trials have yet to incorporate a selected population of cells from bone marrow, several animal studies have detailed their individual capability.

### 3.1.1 Endothelial Progenitor Cells

EPCs arise from a multipotent cell called a hemangioblast and are mobilised from the bone marrow in response to cytokines or ischemia (Penn and Mangi, 2008). EPCs have been shown to increase the capillary density and protect against cardiomyocyte apoptosis after systemic injection in rats with cardiac ischemia (Penn and Mangi, 2008). They facilitate neovascularisation by differentiating into endothelial cells and have been shown to promote *ex vivo* neoangiogenesis that would rescue tissue from ischemia. (Urayama et al., 2008). EPCs can contribute to neoangiogenesis by incorporating to the endothelium of small vessels in the border zone of the infarct. Although they have been shown to transdifferentiate into functionally activate cardiomyocytes *in vitro* this ability has not been reproduced *in vivo* (Badorff et al., 2003).

EPCs do not have an exclusive cell marker, rather there are several markers that identify the EPC population, including CD133, CD34 and vascular growth factor receptor 2 (VEGFR-2 or KDR). However, there is controversy over definition of EPCs by these surface markers as they are expressed in a wide range of cell types. CD34 is expressed on mesenchymal and epithelial cells, and KDR are also widely expressed on a number of blood and endothelial

cells (Hirschi et al., 2008) (Peichev et al., 2000). According to Yoder, EPCs should be defined by their function as circulating cells that have proliferative potential and can differentiate endothelial lineage. They also should have ability to form lumenised capillary-like tubes in vitro and ability to form stable human blood vessels when implanted into tissues (Yoder, 2012). .

For culture and expansion to clinically relevant EPCs can be isolated into 2 different subtypes. Either ‘early EPCs’ or ‘late outgrowth EPCs’ as they appear after a prolonged culture period forming growing in colonies (Navarro-Sobrino et al., 2013). Early EPCs are obtained from peripheral blood mononuclear cells that are seeded onto fibronectin for 4 days in VEGF containing medium, and are CD45, CD14 and CD11b+ akin to a haemopoetic lineage (Fadini et al., 2012). Late outgrowth EPCs have a longer term ability to proliferate and express more endothelial markers such as CD133(Thill et al., 2008). Different subpopulations of EPCs are likely to have a variety of functions. Those expressing CD14 and CD45 may facilitate neoangiogenesis by release of angiogenic factors (Rehman et al., 2003), whereas, CD133 positive cells have higher specifity for an immature cell type, giving rise to greater proliferative potential.

In clinical trials EPCs have been quantified as CD34+/VEGFR2+ or CD34+/VEGFR2+/CD133+. However, cells expressing these surface marker expression are not real endothelial progenitors but instead monocytic progenitors that have angiogenic potential (Rehman et al., 2003). Ziegelhoffer et al concluded that EPCs do not incorporate into vessel walls but support the formation of new vessels by release of growth factors and cytokines acting as perivascular cells (Ziegelhoeffer et al., 2004).

Lack of consensus of what defines an EPC has restricted the translation into clinical practice and remains a work in progress in comparison to other cell populations in the bone marrow (Fadini et al., 2012).

### 3.1.2 Mesenchymal stromal cells

Mesenschymal stromal cells are precursors of non-haemaopoietic tissues that exhibit low immunogenicity. MSCs account for 0.001 – 0.01% of all cells isolated from the bone marrow (Pittenger et al., 1999),. They are an endogenous population that has the potential to differentiate into a variety of cell types of the mesoderm, endoderm, and ectoderm. They can be distinguished from other bone marrow cells due to their adherence to cell culture dishes.

MSCs are characterised by their expression markers as they are positive for Sca1+, CD44, CD73, CD90, CD105, CD166 and STRO-1, but do not express typical markers of HSC such as CD45, CD34 and CD14 (Brooke et al., 2008). Also they have been shown to acquire markers of cardiomyocytes and mediate connections via by connexion 43 gap junctions (Novotny et al., 2008). However, these defining characteristics are becoming outdated as recent studies have shown fibroblasts and perivascular cells express the same markers and have similar gene expression profiles (Covas et al., 2008b),. The International Committee of Cellular Therapy (ISCT) has reiterated the need to quantify the functional potency of these cells using standardised assays (Krampera et al., 2013)

Treatment of ischemic hearts with MSCs has led to increased vascular density (Silva et al., 2005). MSCs may contribute to early angiogenesis by promoting endothelial cell migration and proliferation through paracrine effects. *In vitro* studies have shown MSCs to function as perivascular precursor cells stabilising venous arterial cells (Duffy et al., 2009). They can also contribute towards later stages of angiogenesis in vessel stabilisation. Other *In vitro* studies have observed that MSCs promote EC-EC interactions in non-contact co-culture decreasing endothelial monolayer permeability (Pati et al., 2011),

Studies that have monitored the final location of MSCs to determine migratory paths after transplantation showed that <5% are actually found in the heart, with most becoming immobilised in the lung (Wang et al., 2011). However despite this they still produce beneficial responses in MI patients (Lee et al., 2009a) These trapped MSCs have been shown to express a 28 fold increase in TNF stimulated gene 6 protein (TSG-6) (Lee et al., 2009a). Immunomodulatory factor TSG-6 inhibits neutrophil filtration and protease production. It does so by entering the circulation, reducing the inflammation caused after ischemic injury. By reducing the neutrophil invasion, their negative impact is reduced and hence so is the size of the final infarct. This observation suggests a non-regenerative immunomodulatroy role for cells.

The ability of MSCs to adhere and expand on tissue culture plastics enables their isolation from bone marrow and under set *in vitro* conditions they can differentiate into cardiomyocytes, characterised by their expression of cardiac troponin T, and phospholamban (Song et al., 2010). They are also able to differentiate into endothelial cells after administration of vascular endothelial

growth factor (VEGF) and 5-aziothioprim (5AZT). Both of these differential lineages could be exploited for therapeutic benefit for cardiac repair, and observations in pig, mouse and rat following MI suggesting consistency is conserved between the species (Shake et al., 2002). However, in a swine model allogenic MSCs only transiently increased cardiac function despite contributing to neovascularisation and myogenesis (Shake et al., 2002).

Few studies have accessed the signalling pathways activated, and many have chosen to identify factors released. For instance, Canonical Wnt signaling is retains self renewal and represents a stem cell niche signal. When activated this can cause MSC lineage commitment, whereas proliferation is driven when inhibited. MSCs overexpressing a Wnt modulator, secreted frizzled-related-protein 2 (sFRP2), increased angiogenesis, enhanced engraftment and significantly reduced final infarct size when transplanted into a rat infarcted heart (Alfaro et al., 2008).

### 3.1.3 Haematopoietic Stem cells

HSCs found in adult bone marrow are multipotent stem cells that can produce cells of the lymphoid and myeloid lineages (Yilmaz et al., 2006). They are already routinely exploited in clinical practice for bone marrow transplants. They are characteristically small size and lack lineage markers (lin-). However, due to the many subpopulations there are issues with characterisation using surface expression markers. For example, CD34 and CD38 are commonly expressed in HSCs, but there are some HSCs that are CD34- /CD38-. CD34 expression can be used to distinguish between short term and long term HSCs, with CD34+ applying to short term, and CD34-

distinguishing long term HSCs. Both Lin-CD34+CD38- and Lin-Sca1+ HSCs have demonstrated intercellular connections with resident cardiomyocytes when intravenously injected (Ishikawa et al., 2006; Orlic et al., 2001). However, they have not been shown to differentiate into cardiomyocytes or endothelial cells in the infarcted myocardium.

Mobilisation of these cells is enabled by conditioning with G-CSF (Fu and Liesveld, 2000). This increases matrix metalloproteinase-9 (MMP9) and several other matrix proteases that cleave adhesion molecules in the bone marrow stroma, facilitating mobilisation of not only HSCs but also macrophages and monocytes into the peripheral blood. G-CSF also disrupts the SDF-1/CXCR4 axis mobilising the HSCs enabling them to exit the bone marrow. SDF-1 corresponds with it's receptor CXCR4, which enhances adhesion from blood to endothelium (Brooke et al., 2008). Flt3 ligand has also been shown to facilitate interfere with the CXCR4 signally pathway facilitating the mobilisation of HSCs (Fukuda et al., 2005)

There are differences between surface marker expression in mouse and human HSCs. As animal studies are commonly used the differences must be acknowledged when translating into clinical practise. These differences are presented in Table 1

**Table 1 Species differences in surface marker expression of HSCs**

| Human | Mouse |
|---|---|
| $CD34^{+}$ | $CD34^{-/lo}$ |
| $CD59^{+}$ | $SCA\text{-}1^{+}$ |
| Thy/CD90+ | $Thy1.1^{+/lo}$ |
| $CD38^{-/lo}$ | $CD38^{+}$ |
| c-kit/$CD117^{+}$ | $C\text{-}kit^{+}$ |
| $Lin^{-}$ | $Lin^{-}$ |

From studies using murine MI models there is no clear evidence that to drive cardiac regeneration HSCs differentiate into cardiomyocytes or endothelial cells (Balsam et al., 2004; Murry et al., 2004).

A selected cell population has yet to be identified that can home to the injured myocardium, participate in vasculogeneis, and survive long term after engraftment. It could be that more than one cell subset is required, as use of more than one subpopulation may produce additional or synergistic effects. For instance, the engraftment of HSCs is facilitated when co-transplanted with MSCs. In addition, the immunomodulatroy effect of MSCs decreases the incidence of an immune response (Lu et al., 2006). To establish interactions within the ischemic myocardium, MSC and HSCs, may use different surface

adhesion molecules (Ip et al., 2007). This would thereby influence the mechanism for homing and trafficking.

### 3.2 Preconditioning for optimised therapy

The main issue with injecting cells either intravenously, intramyocardially, or intracoronary, is mass cell loss due to apoptosis due to ‘anikosis’ and migration to the liver and spleen (Lu et al., 2009).. Improving the survival of transplanted cells is imperative Freyman et al, showed that only 0.5% of bone marrow cells injected into a mouse infarct heart survived (Freyman et al., 2006b). While the amount of cells administered is identified as a critical factor for cell therapy if only a minute fraction survive, any potential benefit is surely negated. A few studies have established methods of preconditioning the cells to improve survival and cardiac repair (Table 2). Most of these studies have used MSCs, due to their ease of isolation and adherence to cell culture dishes for *ex vivo* analysis.

**Table 2 -Improving cell therapy by preconditioning**

| Study | Modulator | Outcome (comparison of treated and non-treated groups) |
|---|---|---|
| (Hahn et al., 2008) | FGF-2,BMP2,IGF-1 | ↓ Apoptosis; infarct size<br>↑ Engraftment; cardiac function |

| | | |
|---|---|---|
| (Chang et al., 2012), | HSP 70 | ↓ Cardiac repair; cardiac function |
| (Yang et al., 2009) | Simvastatin | ↑ Survival |
| (Pasha et al., 2008) | SDF-1 | ↓ Apoptosis<br>↑ Engraftment; differentiation; cardiac function |
| (Yao et al., 2009) | Lipopolysaccaride | ↓ Fibrosis; apoptosis of infarcted heart<br>↑ Survival; blood vessel density; cardiac function |
| (Wang et al., 2011) | Neuropeptide Y | ↓ fibrosis<br>↑ differentiation; angiomyogenesis; cardiac function |
| (Wisel et al., 2009) | Trimetazine | ↓ Fibrosis<br>↑ LVEF; fractional shortening |
| (Guo et al., 2008) | HGF | ↑ Angiogenesis; cardiac function |
| (Chang et al., 2012) | Hypoxia | ↑ Survival;Angiogenesis<br>↓ Fibrosis; apoptosis |
| (Ray et al., 2008) | Oestrogen | ↓ Fibrosis; remodelling |

IGF-1 has been shown to reprogram Sca1+ cells for pro-survival signalling and cardiomyogenic differentiention. This process is mediated by upregulation of connexion 43(Cx-43), that forms intercellular Cx-43 is important for cytoprotective function by an antiapoptotic role, preventing cytochrome c translocating from the mitochondria to the cytoplasm. Engraftment is also increased, as induced Sca1+ cells enhance cardiomyogenesis due to colocalisation of cx-43 with myosin and sarcomeric actinin representing coupling with exisiting fibers, increasing gap junction formation (Lu et al., 2009). Bone marrow cells pre-treated with IGF-1 prior to injection into the infarct region where shown to have significantly increased survival, as IGF-1 triggers the AKT pathway. IGF-1 transiently activates the PI3K/AKT pathway. Sca+1 cell stem cells are then able to differentiate into cardiomyocytes. Enoki et al have shown that this improves survival of transplanted MSCs by inhibiting the PI3K pathway(Enoki et al., 2009).

Transplanted cells are injected into an environment of high stress decreasing their viability. When Chang et al preconditioned MSCs with Heat Shock Protein 70 (HSP70), a protective protein against stress, these cells where transplanted into an infarcted heart producing anti-apoptotic properties and increased cell viability. This allowed the treated cells to become integrated into the infarcted myocardium (Chang et al., 2009). Results showed an increase in microvessel density and overall heart function.

Another protein found to suppress apoptosis is the cytokine SDF-1. Cells preconditioned with SDF-1, when transplanted into an infarcted heart, displayed increased cell viability, improved heart function with decreased

infarct size and fibrosis, while increasing vascular density and cardiac differentiation (Pasha et al., 2008).

By preconditioning MSCs with lipopolysaccaride (LPS), the efficacy of MSC transplantation after myocardial infarction can be enhanced (Yao et al., 2009). Benefits identified include increased vascular density and cell survival rate. The LPS induces VEGF expression and p-AKT induction increasing the cardiac function due to enhanced vascularisation.

An apparent issue with most cytokine preconditioning is that they are not currently use in clinical practise, so translation of these into man is a long way down the line. However, statins and trimetazidine, 2 established medications can overcome this problem as they have similar effects to these cytokines. Trimetazidine is an anti-ischemic drug for angina treatment. Hearts treated with trimetazidine preconditioned MSCs were found to have a significant reduction in fibrosis, and increased cell viability compared to normal MSCs transplantation (Wisel et al., 2009). Administration of a 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitor, like simvastatin, shows great potential in not only increasing survival, initiates differentiation, increases angiogenesis, reverses ventricular remodelling and reduces infarct size (Yang et al., 2009). Statins mediate the reduction of proinflammatory cytokines and inhibit expression of Bax, an apoptotic protein. Inhibition of apoptosis may also be regulated by activation of Akt/eNOS (endothelial Nitric Oxide Synthase) pathway (Yang et al., 2009). Increasing eNOS production also leads to increased matrix metalloproteinase (MMP) 9 that facilitates stem cell mobilisation. This increases cardiomyoplasty efficacy and hence improve cardiac function after the MSC

transplantation. Other agents that prevent apoptosis in this microenvironment are cyclosporine A and Lysophosphatidic acid (Liu et al., 2009); (Chen et al., 2008).

In addition to increasing transplanted cell survival other experiments have led to the initiation of certain pathways within the bone marrow cells. For instance, growth hormone production is stimulated in Oestrogen treated bone marrow MSCs and EPCs. This caused improve myocardial remodelling when injected into an infarcted rat heart, by increasing capillary density (Ray et al., 2008), .

Another study investigated the infusion of Neuropeptide Y showing that it directly induces adult cardiomyocyte cell cycle re-rentry and enhances the number of differentiated cardiomyocytes from MSC in the infarcted myocardium resulting in improved cardiac function, reduced fibrosis and increased angiomyogenesis (Wang et al., 2009).

The desired outcome of these transplanted cells is to have a beneficial effect within the ischemic region, and improve cardiac function via various mechanisms. However, the microenvironment of the ischemic border zone is devoid of oxygen and nutrients causing cell death. It is logical that a cytoprotective state needs to be induced. This can be mediated by hypoxia preconditioning. By denying the cells oxygen activates the AKT pathway, so cell viability and cell cycle is continued (Mangi et al., 2003). Compared to controls, hypoxic conditioning enhanced the survival, and angiogenic ability of MSCs.

Preconditioning of BMSCs, according to data from these animal studies described, appears a plausible solution to the integral problems and variability observed in clinical trials. Increasing cell viability within the infarct region is a fundamental factor for this.

## 4. Improving cell retention

### 4.1 Stem cell homing and migration

When a myocardial injury occurs signals are released by the cells in the damaged myocardium to repair the injured tissue. The underlying mechanism by which cells from the BM are recruited to site of injury needs to be fully elucidated. Studies with superhealer mice MRL/MpJ (McBrearty et al., 1998) that have shown that myocardium can regenerate by scarless healing. These mice were found to have 3 fold greater quantity of BM derived cells in the heart than non-injured mice or injured WT mice (Heber-Katz et al., 2004). This effect has been reproduced in WT mice by engraftment of BM from MRL/MpJ mice (Bedelbaeva et al., 2004). The increased efficacy may be due to increased homing, trafficking to infarct region, engraftment survival, or a variety of these.

Several chemokines and growth factors are important for cell homing to the infarcted region, including insulin growth factor 1 (IGF1), stromal cell derived factor 1 (SDF1), monocyte chemoattractant protein 3 (MCP3) and hepatocyte growth factor (HGF). The mechanism of recruitment can be exploited for cell transplantation to maximise the amount of cells directed to the infarct region and ensuring their residence. Preconditioning the BMSC to express specific receptors to these paracrine factors has been explored with promising results

using animal models, as previously described. Ip et al investigated the myocardial migration and engraftment of MSC. By preconditioning the cells with G-CSF and stem cell factor (SCF) the myocardial repair was enhanced and cardiac function was increased (Ip et al., 2007). They showed that BM-MSC are dependent on integrin α4β1, α5β1 and CD29 for migration to the ischemic myocardium.

By generating a gene expression profile from the MI heart, chemokines, adhesion molecules and cytokines up regulated by injury have been identified. After MI SDF-1 expression is dramatically increased, which acts as a homing signal to those cells expressing CXCR4 including HSC and MSCs from the bone marrow. This expression is present immediately after, and persists for several days. SDF-1 also improves vascular density, and prolongation of resident SDF1 expression. The SDF-1 gradient is important for stem cell recruitment, so maintenance of the gradient is desired. Lee et al demonstrated that after BMSC transplantation, SDF-1 levels in the myocardium increase (Lee et al., 2009a). Therefore cell therapy generates a favourable chemokine gradient for stem cell recruitment into the infarcted myocardium. SDF-1 mediated recruitment is dependant on the membrane expression levels of CXCR4 receptor. Overexpression of SDF-1 increases stem cell homing and engraftment to ischemic regions. This can also be endogenously upregulated in the myocardium by endothelial nitric oxide synthase (eNOS) derived nitric oxide (NO), promoting migration. While increasing the chemokine increases the homing effect, so does upregulation of the CXCR4 receptor. IGF-1 can up-regulate this expression of CXCR4 (Penn and Mangi, 2008),. This leads to greater survival and homing of MSCs,

and therefore greater improvement of cardiac function through greater paracrine factor expression.

However, SDF-1 is not exclusively expressed in cardiac tissue as it is also expressed in the liver and spleen (Zhang et al., 2008). Therefore, transplanted BMSCs migrate to these regions aswell. By doing so they are eliminated through the pulmonary first pass effect where the cells become microembolised. This may reduce the degree of 'homing' and account for the evidence that only 5 % of transplanted BMSCs are found in the infarct region. Surface marker expression can have an effect on micro-embolism given that since as MSCs with high expression of podocalyxin (PODXL) and CD49f are less prone to form pulmonary embols and increasing numbers are found in the heart compared with PODXL-/CD49f- MSCs (Lee et al., 2009b)

Hypoxia inducible factor 1 (HIF-1) is another cytokine implicated in homing of stem cells albeit indirectly. HIF-1 is released in the ischemic myocardium casing an increase in VEGF expression that stimulates the migration of EPCs (Novotny et al., 2008).

Once the cells home to the ischemic region, unless they are injected directly to the ischemic border zone, migration through the endothelium is imperative. Similar to leukocytes, the recruited stem cells enter the extracellular matrix from the blood by tethering, adhering, and intergrin rolling along the endothelium due to the actions E and P selectin molecules. Integrin β1 has also been identified as crucial for the rolling and adhesion in MSCs (Ip et al., 2007). Migration and homing of MSCs is mediated by integrin β1 via the MCP-3-CCR1 and CCR2 axis (Ip et al., 2007). This is distinct from CD34+

haematopoietic cells that home via the CXCR4 receptor. This suggests that MSCs and HSCs have distinct classes of surface adhesion receptors for interactions with either the ECM or resident cells.

Rolling is instigated by the extracellular expression of P and E selectin on the endothelium surface that complements selectin receptors on MSCs. Firm adhesion to the endothelial cells is then mediated by VCAM-1 and VLA-4 expression. For transmigration integrin α4β1 binds to the endothelial VCAM-1. Migration through the ECM is then mediated by adherence to hyaluronic acid by CD44 and is subsequently facilitated by MMP-2 which cleaves a path for movement along the chemokine gradient (Steingen et al., 2008).

## 4.2 Adhesion

Once the cells have been able to home to the infarct region, it is vital for the BMCs to attach and remain within this region. When cells are transplanted into the heart if they do not engraft anoikis will occur (Michel, 2003),. Anikosis is programmed cell death that occurs due to lack of attachment, or loss of contact with, ECM proteins. For cells to engraft, they need matrix support. If there is inadequate interaction between glycoproteins of the extracellular matrix (ECM) and the cells, apoptosis will occur. This interaction ensures the survival of the endogenous adhered differentiated cells within the heart. Therefore, adhesion is required involving integrins, connexins and adherins.

Integrins are an important adhesion mediators and attachment via integrins causes tensegrity, a tensile stress within the cell, preventing apoptosis (Song et al., 2007). However, if cells do not attach the opposite will occur, and apoptosis will not be repressed, causing the cells to die.

In addition to attachment, integrins are fundamental for all cell-matrix interactions including firm adherence and migration. Integrin activation is required for these interactions to occur, not simply surface expression. α5β1 is important for firm adherence to fibronectin, but only in it's activate state. The α5 integrin unit is also involved with migration to fibronectin, via Neuropilin 1 (NRP1). NRP1 binds to active α5 integrins, via the GAIP interacting protein C terminus (GAIP) mediating endocytosis for re-presentation on the leading edge of migration (Simons, 2012). Fibronectin is upregulated after infarction therefore a similar mechanism could be involved in the migration and adhesion of stem cells to the ischemic myocardium (van Dijk et al., 2008). NRP1 is recognised as important for endothelial cell adhesion to fibronectin and therefore may have an important role for MSCs too (Valdembri et al., 2009).

NRP-1 is a transmembrane glycoprotein that acts as a co-receptor for VEGFRs and SEMA3A that regulates vascular and neural development (Zachary, 2011). Mice with targeted NRP1 disruption have reduced blood vessel growth, particularly in the spine and brain (Fantin et al., 2013). Fantin et al, also showed identified the role of NRP1 in postnatal angiogenesis and arteriogenesis in the heart, with fewer coronary arteries and capillaries in the hearts of mice with mutated NRP1 compared to control hearts (Fantin et al., 2014). NRP1 and VEGFR2 only interact in the presence of VEGF-$A_{165}$ activating signalling pathways involved in migration and angiogenic sprouting (Soker et al., 2002). However, NRP1 can function independent of VEGFR2 supporting VEGF mediated endothelial cell migration through PI3K/Akt signaling in tumour cells not expressing VEGFR2 (Bachelder et al., 2001).

Similarly MSCs express NRP1 but not VEGFR2. NRP1 in MSCs been shown to be an essential co-receptor with Platelet Derived Growth Factor Receptors (PDGFRs), particularly PDGFRα (Ball et al., 2010). This study identified the interaction between NRP1 and PDGFR as indispensable for migration of MSCs towards PDGF-AA & PDGFR-BB.

## 4.3 Mechanism of action

While the proposed mechanism of action behind benefits of transplanted BMSCs have yet to be fully deciphered, evidence is building that it is due to release of paracrine factors (Drexler and Wollert, 2009). The frequency of stem cell engraftement and transdifferentation detected is too low to have a significant impact. There is currently no evidence from human trials of transdifferentation to cardiomyocytes. Increasing microvasculature and neoangiogenesis, in addition to enhanced cardiomyocyte survival is often cited to be the basis of BMSC effects(Clifford et al., 2013). Also, transplanted cells found trapped in the lungs still proved to decrease infarct size by an immunomodulatory mechanism (Lee et al., 2009a). As such, release of cytokines and growth factors by transplanted BMSCs has been proposed as a major feature contributing to cardiac regeneration and repair (Figure 5).

Approximately 40 of these secreted factors have the potential for cardiac repair, including VEGF, BMP2, IGF-1 and sFRP2 (Gnecchi et al., 2008a). Some researchers have suggested that delivery of an optimised variety of

growth and cytokine factors could be all that is necessary post MI to exert the same benefit as administration of BMSCs. (Mirotsou et al., 2011). However, paracrine effects are not the only mechanism involved

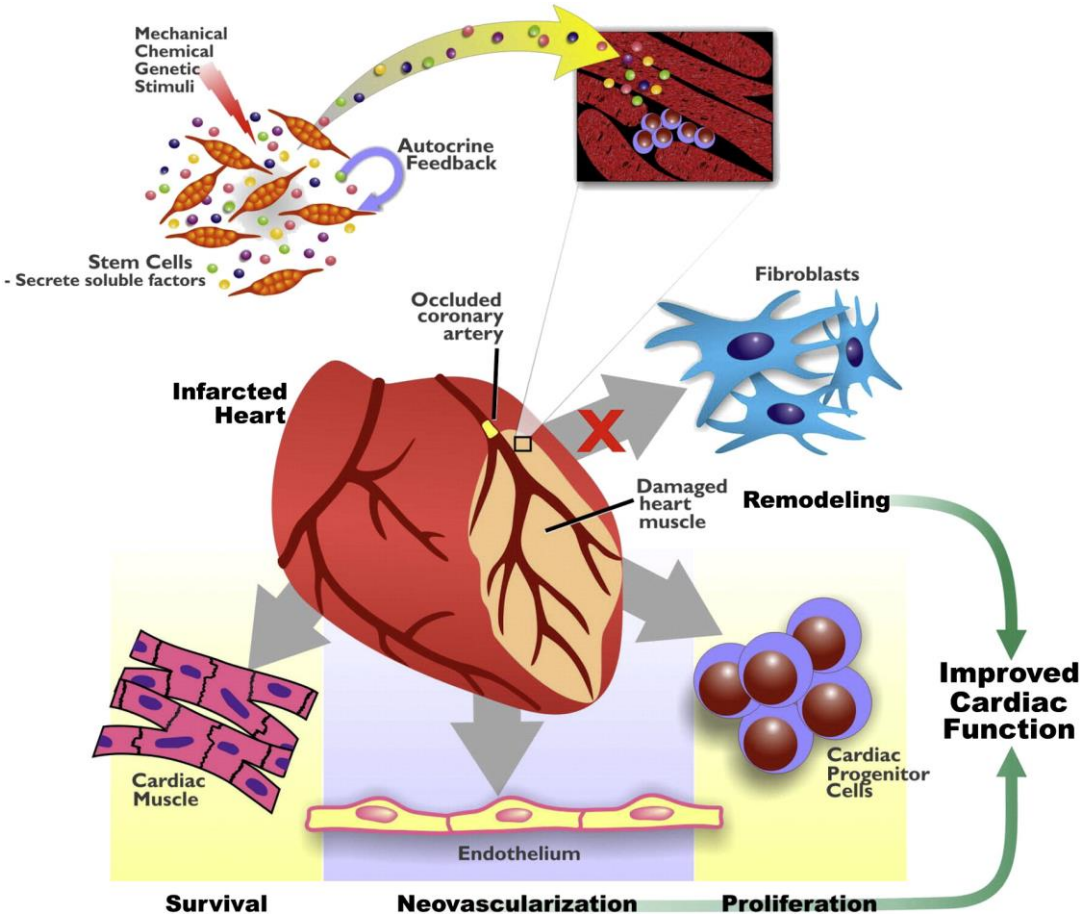



Figure 5 Proposed paracrine mechanisms for cardiac repair (Gnecchi et al., 2008b)

In response to hypoxia BMSC release cytokines and growth factors induce signalling pathways leading to cardiac repair

### 4.3.1 Neovascularisation

Restoration of vascular supply to ischemic myocardium is vital as chronic ischemia after MI will cause negative ventricular remodelling that leads to

heart failure. With the occlusion of the coronary artery, new vasculature can be formed by neovascularisation involving angiogenesis, arteriogenesis and vasculogenesis. The processes of angiogenesis and arteriogenesis involves growth factors including VEGF, bFGF and HGF. This leads to endothelial cell migration, vessel enlargement and extracellular matrix synthesis. BMSCs express these proangiogenic factors. A study injecting BM-MNCs into ischemic swine heart showed the cells not only expressed bFGF, VEGF and angiopoietin-1, but levels of interleukin 1β and TNFα in the myocardium were also increased after BM-mononuclear cell administration (Kamihata et al., 2001). This resulted in an increased capillary density and localised blood flow to infarct region 3 weeks after initial coronary ligation. These secreted factors are likely to have contributed to stimulate angiogenesis. It has been reported that supernatant derived from cultured BMCs administered to MI mice has shown to have pro-angiogenic effects and protection against CMC necrosis and apoptosis (Korf-Klingebiel et al., 2008).

### 4.3.2 Myocardial protection

An important mechanism for reduction of infarct size is to improve cardiomyocyte survival after infarction. It is vital that this is an immediate effect in the ischemic region. The Akt-1 pathway is important in the release of cytoprotective proteins (Gnecchi et al., 2005). Gnecchi et al showed that by injecting cultured medium from Akt overexpressing cells into the infarct border, large reduction of cardiac apoptosis and infarct size was observed compared to medium from native MSCs. Subsequent studies idenitified

VEGF, bFGF, HGH, IGF-1 and thymosin B4, as upregulated in the Akt overexpressed MSCs after being exposed to hypoxia. Native MSCs show the same upregulation of these proteins but to a lesser extent than the Akt-MSCs. IGF-1 in particular prolongs the survival of the cardiomyocytes whereas another cytokine released by MSCs, Tumour Necrosis Factor α (TNF α), protects the CM against hypoxic damage (Meldrum, 1998).

HGF has been associated with MSC migration, but there is also evidence that HGF imparts pro-survival effects on cardiomyocytes within the infarct zone (Gude et al., 2008),. HGF is the ligand for the receptor c-met that activates the PI3K/Akt pathway (Figure 6). This increases notch activation that leads to further akt phosphorylation. Gude et al have identified that activated overexpression of the notch pathway in infarcted hearts decreased the infarct size and increased anterior wall thickness, showing that not only does notch provide pro-survival effects but also stimulates CM proliferation.

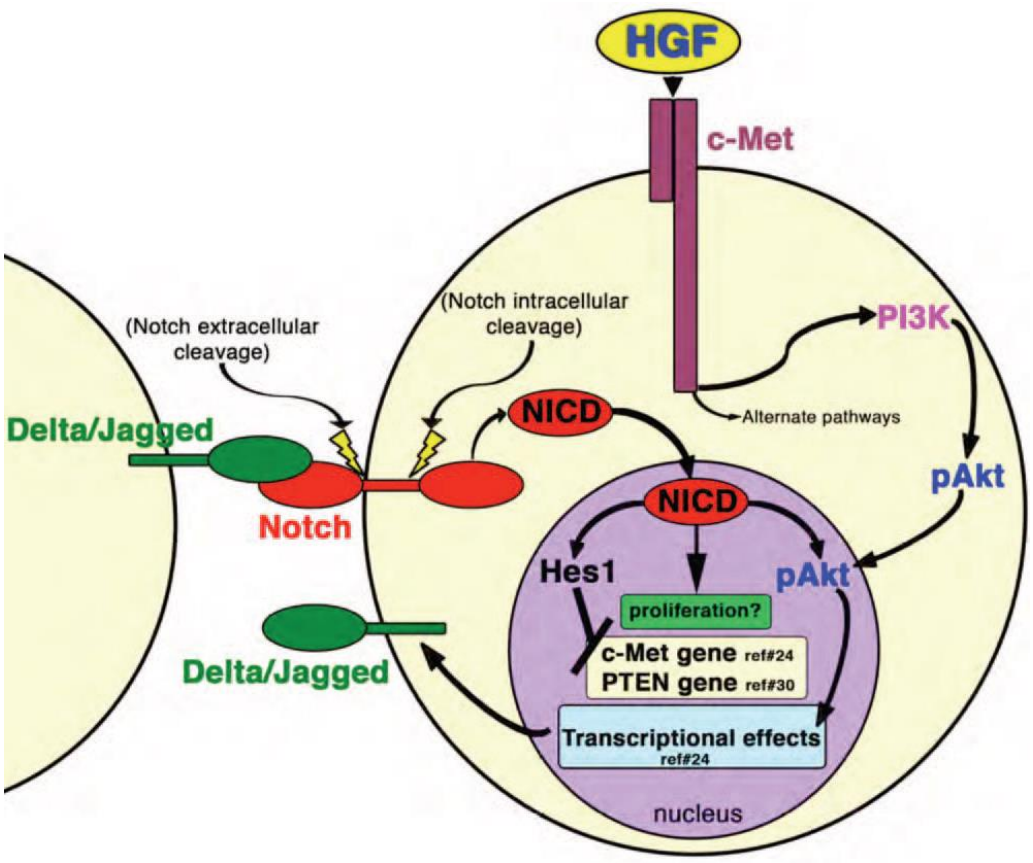



**Figure 6 Notch and HGF/c-met signalling pathways (Gude et al., 2008)**

### 4.3.3 Immunomodulation

Not all BM cells administered are contained to the heart, they are often found in several other organs, mainly in the lung. Lee et al studied MSCs microembolised in the pulmonary vasculature. This caused major changes in the gene expression upregulating an anti-inflammatory protein TSG-6. TSG

inhibits neutrophil migration into sites of inflammation and binds to hyaluronan fragments inhibiting their inflammatory effects.

### 4.3.4 Cardiac remodelling

Modulation of the post infarct scar can be altered by paracrine factors released from transplanted cells. Alteration of the extracellular matrix by decreasing collagen types I and III and tissue inhibitor of metalloproteinase 1 reduces the infarct expansion. Injection to the border region of infarct myocardium has shown to decrease fibrosis and apoptosis while increasing myocardium thickness. Although there is no regeneration, cardiac function is preserved (Berry et al., 2006).

### 4.3.5 Cardiac regeneration

The ability to regenerate infarcted myocardium is the gold standard for cellular therapy. However, there is very little evidence to suggest that transplanted BMSC are transdifferenting to cardiomyocytes in the infarcted border region. However, they may indirectly induce cardiac stem cells. Trophic growth factors HGF and IGF-1 have been shown have been shown to induce migration, proliferation and transdifferentation of cardiac stem cells. MSC release both of these proteins so may be recruiting CSCs when injected into the infarct area. Even at mRNA level HGF and IGF-1 was significantly upregualted in human BMSC treated hearts compared to controls (Yoon et al., 2005). The role on endogenous regeneration after BMSC treatment

needs further investigation. While MSCs have showed promising results, hearts treated with HSCs, have not shown significant proliferation of CSCs or cardiomyocytes. In vitro work with EPCs has identified their ability to induce migration of c-kit+ cardiac progenitors (Urbich et al., 2005)

It is probable that BMSC repair myocardium through several pathways involving myogenesis and angiogenesis. The model currently suggested involves the release of growth factors by transplanted stem cells that increase angiogenesis or aide in the survival of cardiomyocytes in the infarct region. Clinical trials have given modest results, but shown potential for development. There is great scope to improve this therapy by preconditioning the cells and selection of an individual cell population to create a more targeted cellular therapy to infarcted myocardium.

## Research question

Can the adhesion and angiogenic functionalities of bone marrow derived stem cells be enhanced by preconditioning strategies in an *in-vitro* model?

## Hypothesis

The adhesion and vascular support capacity of bone marrow derived stem cells can be enhanced by pharmacological preconditioning

## Aims and Objectives

The bone marrow contains a very broad range of cells, and the aim of this project is to investigate if these bone marrow cells can be preconditioned to achieve a better therapy.

1. To characterise the cells in the BM-MNC fraction, that are being used in clinical trials;
2. Develop *in-vitro* assays to characterise their functional quality attributes associated with engraftment and angiogenesis capabilities, in conditions that mimic the environment of the ischemic heart
3. To explore strategies to improve the retention & pro-vasculogenic potential of these bone marrow stem cells in an *in-vitro* model

To do this I will be using bone marrow aspirates of patient samples from the REGENERATE-IHD clinical trial (NCT00747708) and healthy donor aspirates from University College Hospital.

# Chapter 2 – Materials and methods

The following methods are applicable to all chapters; all other methods and materials are further described in appropriate chapters.

## 1. Cell culture

All cell culture was carried out in laminar flow cabinets that had been pre-cleaned with 70% ethanol. Aseptic technique was used to minimise risk of contamination

### 1.1 Bone Marrow mononuclear cell isolation

Bone marrow aspirates were obtained from healthy human donors, kindly donated by University College London Hospital. 1 part whole bone marrow (WBM) was diluted with 1 part Phosphate Buffered Saline (PBS). The mononuclear cell (MNC) fraction was obtained using a Ficoll density gradient.

20ml of WBM was carefully pipetted onto 20ml of Ficoll-PAQUE PLUS (GE Healthcare;17-1440-02) in 50ml Falcon Centrifuge tubes. This was centrifuged at 650g for 30 minutes at 20°C with the brake/acceleration set to 0. A band of MNCs is observed as an orange cloudy band above the clear ficoll and below dark red plasma. This MNC layer was removed with a 5ml pipette. The MNC fraction was washed with 3 parts dPBS to 1 part MNC and then centrifuged at 650g for 15 minutes at 20°C. The supernatant was

discarded and cell pellets resuspended in 10ml of hMSC growth media (as described below). Cells were used in experiments or used to be seeded for mesenchymal stromal cell culture

### 1.2 Media

Human Mesenchymal Stromal Cells were maintained in Dulbecco's modified Eagles medium (DMEM) low glucose (1g/L) plus Glutamax$^{TM}$ (Invitrogen; 10567-014), 1ng/ml recombinant basic Fibroblast growth factor (bFGF; R&D systems) and 10% v/v Fetal Bovine Serum (FBS; SLI, EU-000-F). Before use media was filtered using Millipore Express pore plus. Media composition was chosen from that of previous work on MSCs in the laboratory.

Human Umbilical Cord Endothelial Cells (HUVECs) were maintained in EGM-2 plus Bulletkit (Lonza; CC-3162).

### 1.3 Mesenchymal Stromal Cell Culture

After BM-MNC isolation (see §1.1) cells were counted using a haemocytometer and seeded at a density of 1 x $10^5$ cells/$cm^2$ in Nunc T75 tissue culture flasks (Nunc, Stafford, UK) and maintained in a 5% CO2 humidified incubator. Media was replaced after one day and then subsequently every 3 days. hMSCs were ready for passaging at 80% confluency.

### 1.4 Cell Passaging

Cells were passaged at ~80% confluency, unless stated. To passage all media was aspirated from tissue culture flask, and cells washed once with

0.1ml dPBS/$cm^{2.}$ 0.25% Trypsin-EDTA (T/E; Invitrogen; 25200-056) was added in quantities as per Table 3. Flasks were incubated at 37°C for 3 minutes and checked using a light microscope for cell detachment. The T/E was quenched with FBS containing growth media to neutralise the enzyme activity. Resultant cell suspension was transferred to a centrifuge tube and centrifuged at 300g for 3 minutes. Supernatant was then discarded and the cell pellet was resuspended in an appropriate volume of growth media. hMSCs and HUVECs were seeded at of 1 x $10^4$ cells/$cm^2$. Total volume for each culture vessel was made up to volume stated in Table 3 and media changed every 3 days. HUVECs were used up to passage 4.

| Flask size | Media volume (ml) | Trypsin Volume (mL) |
|---|---|---|
| T75 | 12 | 2 |
| T25 | 5 | 1 |
| 6 well | 2.5 | 0.5 |
| 12 well | 1.5 | 0.3 |
| 24 well | 1 | 0.2 |
| 48 well | 0.5 | 0.1 |

**Table 3 - Appropriate volumes of trypsin and Media or specific cell culture dishes**

## 1.5 Cell Counting

After centrifugation and resuspension cells could be counted using a haemocytometer. A haemocytometer glass slip was placed onto the haemocytometer with Newton's rings formed between the slip and the haemocytometer slide. The cell suspension was pipetted up and down to ensure homogenous distribution and 10µl of the cell suspension was pipetted the top and bottom chambers under the glass slip. The total number of cells in the four corner square, each containing 16 smaller squares. For each chamber the total number of cells was averaged for each square (ie.4) and multiplied by $1x10^4$. This gives the number of cells per ml since the volume of a square is $0.1mm^3$. This value and the volume of cell sample was used to calculate total number of cells in the cell sample.

## 1.6 Storing Cells

A working cell bank of MSCs was created from various isolations to serve as allow experiments to be repeated using the same isolation at different time points. To store cells they were first trypsinsed as described above. After centrifugation and aspiration of supernatant the cell pellet was resuspended in 10% Dimethyl sulfoxide (DMSO; Sigma; D5879) and 90% FBS. Cell concentration was adjusted to $1 \times 10^6$ cells/ml, and 1ml was added to each cryovial (Nuncleon). Cyrovials were then placed into a freezing container (Nalgene; 5100-0001) and then into a -80°C freezer to gradually reduce the

temperature, by 1°C per minute, so the cells are not shocked, or stressed. The following day the cyrovials were placed into liquid nitrogen for long term storage.

### 1.6 Thawing cells

Required cells were removed from liquid nitrogen storage containers, and placed into a liquid nitrogen containing dewar until the thawing process. Cyrovials were thawed in a 37°C water for 2 minutes and gently resuspended before adding drop wise to 4ml pre-warmed growth media. This was centrifuged at 300g for 3 minutes and 21°C and cells were plated as per §1.3

### 1.7 Mycoplasma testing

To ensure all cultures were negative for mycoplasma, a small scrapping was made on the cell monolayer. 1ml of media was collected from the same flask and sent to Surrey Diagnostics (Cranleigh, UK) to analyse the samples for mycoplasma

## *2. Immunocytochemistry*

Cells were fixed with 4% w/v paraformaldehyde (PFA) for 15 minutes. Fixed cells were then washed twice with dPBS. In order to eradicate non-specific interactions the cells were blocked using a blocking solution of 10% serum of species the secondary antibody is hosted from, and 0.25% v/v triton-X, for 40 minutes at room temperature. The blocking solution was aspirated, and the

primary antibody, at specified dilution was added to the well, and incubated overnight at 2-8C. This was then washed twice with dPBS and then secondary antibody at specified buffer was added and incubated for 1 hour, and before it was washed three times with PBS. A 1/10000 dilution of 4,6 diamidino-2-phenylinode (DAPI) solution for 3 minutes and then washed once with dPBS and once with $dH_20$. Fluorescence images were taken using a Nikon Eclipse TE2000-U fluorescence microscope, and analysed with NIS-element software

*Primary and secondary antibodies*:

| Host | Antibody | Reactivity | Company | Catalogue No | Dilution |
|---|---|---|---|---|---|
| Mouse | IgG | HCAM | Millipore | CBL154 | 1:500 |
| Mouse | IgG | THY-1 | Millipore | CBL415 | 1:500 |
| Mouse | IgM | STRO-1 | Millipore | MAB4315 | 1:500 |
| Mouse | IgG | MCAM | Millipore | MAB16985 | 1:500 |
| Mouse | IgG | CD14 | Millipore | MAB1219 | 1:500 |
| Mouse | IgG | CD19 | Millipore | MAB1794 | 1:500 |
| Mouse | IgG1 | PECAM | R&D | MAB2148 | 1:400 |
| Mouse | IgG | SMA | R&D | | |

| | | | | | |
|---|---|---|---|---|---|
| Goat | IgG | NRP1 | R&D | AF566 | 1:100 |
| Chicken | IgG Alexa Flur 488 | Goat IgG | Invitrogen | A-21467 | 1:200 |
| Goat | IgG Alexa Flur 488 | Rat IgG | Invitrogen | A-11006 | 1:1000 |
| Goat | IgG Alexa Flur 555 | Mouse IgG | Invitrogen | A-11001 | 1:1000 |
| Goat | IgG Alexa Flur 488 | Mouse IgM | Invitrogen | A-21042 | 1:1000 |
| Goat | IgG Alexa Flur 488 | Rabbit IgG | Invitrogen | A-22008 | 1:1000 |
| Goat | IgG Alexa Flur 555 | Rabbit IgG | Invitrogen | A-21428 | 1:1000 |

Before each antibody was used to ensure accurate interpretation of results, negative, and where possible, positive controls were used.

No primary antibody control – To test any possible background emittance from the secondary antibody alone

Isotype Control – Samples were incubated with the Mouse IgG negative control (CBL10, Millipore, UK), using the same method as described above,

and then incubated with the desired secondary antibody, before being viewed using the fluorescence microscope. This ensured background staining was negligible.

***3. Characterisation of MSCs***

MSCs were confirmed according to the current ISCT definition ie. Their adherence to tissue culture plastic; specific surface antigen expression of CD105+, CD90+, CD73+, CD19-, CD14- and trilineage differentiation potential to osteoblasts, adipocytes and chondroblasts. These assays were carried out as described

### 3.1 Trilineage Differentiation

#### **3.1.1** Osteogenic differentiation

MSCs at stated passage and experimental group were seeded into a 12 well plate for classical stain differentiation, and a T25 for a gene expression study, both at a seeding density of 5 $x10^3$ cells/$cm^2$ with normal MSC growth media. After 2 hours incubation at 37°C media was replaced by Complete Osteogenic Differentiation Media (Invitrogen, UK). Gene expression studies were harvested after 24 hours. Otherwise, cultures were refed every 4 days. After 21 days media was removed and cells were fixed with 4% paraformaldehyde solution for 15 minutes. After fixation the wells were rinsed with distilled water and stained with 2% Alizarin Red S solution (pH 4.2; Sigma) for 3 minutes. Wells were rinsed 3 times with distilled water and visualised under a light microscope

### **3.1.2** Adipogenic differentiation

MSCs at stated passage and experimental group were seeded into a 12 well plate for classical stain differentiation, and a T25 for a gene expression study, both at a seeding density of 1 x$10^4$ cells/$cm^2$ with normal MSC growth media. After 2 hours incubation at 37°C media was replaced by Complete Adipogenic Differentiation Media (Invitrogen, UK). Gene expression studies were harvested after 24 hours. Otherwise, cultures were refed every 4 days. After 14 days media was removed and cells were fixed with 4% paraformaldehyde solution for 15 minutes. After fixation the wells were rinsed with distilled water and stained with 4% Oil Red O solution (Sigma) for 5 minutes. Wells were rinsed 3 times with distilled water and visualised under a light microscope

### **3.1.3** Chondrogenic differentiation

MSCs at stated passage and experimental group were harvested and generated to a cell concentration of 1.6 x $10^7$ cells/ml. A micromass culture was generated by seeding 5ul droplets of cell solution into the centre of a 6 well plate well. After incubating micromass for 1 hour 2ml warmed chondrogenesis media (Invitrogen,UK) is added. Media was replaced every 3 days. After 14 days media was removed and cells were fixed with 4% paraformaldehyde solution for 15 minutes. After fixation the wells were rinsed with distilled water and stained with 1% Alician blue (Sigma, UK) prepared in 0.1N HCl for 30 minutes. Wells were rinsed 3 times with 0.1 HCL then

distilled water to neutralise the acidity, and observed under the light microscope.

### 3.2 Surface marker expression

MSCs, at stated passage, were grown to 80% confluence and prepared for immunocytochemsitry as according to section 2. Fixed cells were probed for CD44, CD90, CD105, CD14 and CD19

## *4. Quantitative Polymerase Chain Reaction*

Gene expression was quantified using qualitative polymerase chain reaction (qPCR). RNA was isolated from harvested cells using Qiagen RNeasy kit (Qiagen, UK). In brief cells pellets were resuspended using RLT buffer and centrifuged in a QIAshredder at full speed for 1 minute to homogenise the cell suspension. Then RNA was isolated according to the manufacturer's instructions. RNA was eluted from the column using 14μl of RNase-free water and quantified using a Nanodrop. cDNA was then synthesised using Reverse Transcription kit following manufacturer's instructions. For each reaction a master mix was made up of 1μl Quantiscript reverse transcriptase, 4 μl RT buffer, and 1 μl of RT primer mix, which was added to 14ul of template RNA. In a thermocycler this was incubated at 42ºC for 15 minutes, followed by 3 minutes at 95ºC

Resulting cDNA was used with SYBR green Quantitect (Qiagen, UK) and Quantitect Primers. This was detected using CFX Connect Real time PCR Detection system (Bio-Rad, UK). The cycling conditions for the two step RT-PCR were as follows:

PCR initial activation step – 15 minutes at 95°C

*3 Step cycling, repeated for 40 cycles :*

1. Denaturation – 15 secs at 94°C
2. Annealing – 30 secs at 55°C
3. Extension – 72 secs at 72°C

Cq values obtained for each gene were normalised to the housekeeper gene, GAPDH.

## *5.* In-vitro *functional assays*

Throughout the thesis the same optimised in vitro assays are used to access static adhesion, migration and angiogenic potential, except where specified.

### 5.1 Static adhesion

Cell adhesion assays were performed in 48-well plates that were coated with 20 ng/ml fibronectin (R&D Systems). Wells were then washed three times with PBS and blocked with 2% BSA in PBS for 1 h at 37°C. Then, 2% Bovine Serum Albumin (BSA; Sigma Aldrich) in PBS alone-coated wells was used as negative control. 1 x $10^4$ MSCs, at passage 3 or 5, or BM-MNCs per well were seeded on fibronectin-coated plates and cells were incubated in hMSC media for 5, 20 and 40 mins at 37°C. Non-adhered cells were aspirated and

wells were washed twice with PBS. Cells were photographed using a Nikon Eclipse TE2000-U microscope for assessment of adhesion at x 4 magnification in 3 random fields of view.

### 5.2 Chemotaxis

Chemotaxis of hMSCs and BM-MNCs were performed in 24 well plates (BD; 353503) with 8-µm pore cell culture insert (BD; 353182). Serum free media was added to the lower well and insert and incubate for 1 hour to allow equilibrium. The cells were suspended in serum-free DMEM + Glutamax at a concentration of 3 x $10^5$/ml. Then, 1 x$10^5$ cells in 400 µl/well were loaded onto the upper chambers. The lower chambers were filled with serum-free DMEM + Glutamax containing 0 or 100 ng/ml SDF-1. After 3 hours incubation at 37ºC, the non-migrating cells were aspirated and then gently scraped from the upper membrane using cotton swabs. The membranes were fixed in 2% paraformaldehyde for 10 minutes and washed twice in PBS. The migrated cells were stained with 0.5% crystal violet (Sigma, UK) in 5% ethanol for 30 mins. The membrane was mounted on a microscope slide and migrated cells counted in three random fields of view at x 10 magnification. Cell counting was performed using ImageJ, all counts were nromalised to the mean of the control ie. Number of cells on membrane with serum free media in lower well. This is known as the migration index.

For migration of MNCs, as the cells are non-adherent, the cells do not attach to the membrane and locate to the bottom of chamber where they are fixed, stained and identified as above.

### 5.3 Tubule formation

The ability of MSCs to form tubule like structures was assessed using an in vitro angiogenesis assay(Salani et al., 2000). 50µl Matrigel Growth Factor Reduced (BD; Cat.no. 354220) was added to wells of a precooled 96 well plate and incubated at 37°c for 1 hour. $1 \times 10^4$ hMSCs, or BM-MNCs, in 150µl EGM-2 media per well were seeded onto the Matrigel.

Cells were then incubated for 18 hours and vessel branch formation was counted using ImageJ software. The structures were then fixed in 4% Paraformaldehyde (PFA) for 10 mins and washed 3 times in PBS, then blocked with 10% Goat Serum (Invitrogen,UK) for 30 minutes. Cells were then immunolabelled with 1/1000 diluted, mouse anti-PECAM1 (Millipore, UK) and 1/200 mouse anti-αSMA (R&D Systems, UK) overnight at 4°C. These were treated with 1/1000 Alexa Flour 488 Goat Anti-mouse antibody for 1 hour, followed by 3 minutes treatment of 1/1000 DAPI for nuclei staining.

Human Umblical Cord Vein Endothelial Cells (HUVECs) were used as a positive control.

### 5.4 Vascular support assay

To assess the ability of MSCs to support a pre-existing network of endothelial cells they were seeded onto a pre-formed network of HUVECs and observed over the next 24 hours.

### 5.5 Hypoxia

To mimic the ischemic environment after infarct all experiments were repeated in a hypoxic environment. Plates were placed inside a custom made humidified hypoxic chamber purged at 2% oxygen to mimic an ischemic environment. Manufacture and validation of the chamber, as previously described (Mondragon-Teran et al., 2009)

## *6. Statistical* analysis

All experiments were performed in triplicate with at least 3 different isolations of MSCs or MNCs. Data is expressed as mean ± SEM. Differences were analysed by Analysis of Variance (ANOVA). A P-value less than 0.05 was considered statistically significant. Microsoft Excel and SPSS software were used to perform the statistical analysis

# Chapter 3 – Engraftment response of Bone marrow stem cells to preconditioning with SDF-1

## Introduction

Traditional priming strategies for stem cells are concentrated on increasing the survival of transplanted cells, but this is unlikely to increase efficacy post injection due to intrinsic MSC biodistribution. This may be enhanced by optimising adhesion and migration to target the intended region of interest ie. Infarct myocardium. The advantage of this is that it avoids complex delivery to such tissues and will reduce the number of cells required for therapeutic application. Stimulation with stromal derived factor 1 (SDF1) is one such strategy that will be investigated in this chapter.

SDF1 is a chemokine of the CXC family that interacts specifically with CXCR4 and CXCR7, G-protein coupled receptors (Nagasawa et al., 1994). SDF1 and CXCR4 are expressed in many cell types and tissues. SDF-1 is over expressed on myocardial infarction, however this is only short term and decreases after 7 days (Askari et al., 2003). SDF-1 mediates a response via CXC4R increasing the expression of VEGF-A and further. In endothelial cells stimulation with SDF-1 has been reported to up regulate α1, 4 and 5 integrins and increased adhesion (De Falco et al., 2004). Integrin α5β1 is involved in firm adhesion to fibronectin, an extracellular matrix protein that is expressed in the ECM after myocardial infarction, therefore stimulation with SDF-1 would suggest improved

engraftment within a post infarction environment (Zhang et al., 2008). Integrin β1 (CD29) is important for not only adhesion, but also migration and blockade has shown to significantly decreased percentage of cells adhered (Ip et al., 2007). Adhesion isn't the only functionality of the cell that is modulated, SDF-1/CXCR4 signalling also activates PI3K/Akt pathway that contributes towards cell survival, proliferation and migration (Kucia et al., 2004). Pasha et al demonstrated *in-vitro* and *in-vivo* models for use of MSCs by stimulation with SDF-1 in a rat infarct model. They concluded this was primarily due to the activation of this Akt pathway by SDF-1.

In the current study, we assessed the effect of SDF1 pre-conditioning on hMSCs and BMCs *in vitro* to determine whether functional cell characteristics, attachment and migration, both of which are required for cell retention, are improved. Experiments were performed using hMSCs at passage 3, reflective of the typical *in vitro* passage number at which an hMSC product might be obtained; and passage 5, to reflect the limit of acceptability of hMSC products based on literature reports of proliferation rate, clonogenicity and differentiation potential (Bertolo et al.; Madeira et al., 2012

). Subsequently, we performed experiments using passage 3 hMSCs in hypoxia (2% oxygen) to determine functional characteristics in conditions that more closely mimic the physiological oxygen tensions

## Hypothesis

Stimulation of human bone marrow derived MNCs and MSCs with SDF-1α will increase their adhesion, migration and angiogenic potential.

## Method & Materials

All methods and assays are as described in Chapter 2, except the assays were not performed in hypoxic conditions for MNCs due to the limited of cell samples obtained from the REGENERATE trial (Table 1).

**Table 4 – Patient medical details of Bone Marrow Mononuclear cells used**

| Patient ID | Trial | Hypertension | Hypercholesterolemia | Smoking status | NYHA class |
|---|---|---|---|---|---|
| **X31** | DCM | No | No | Non | II |
| **X107** | IHD | No | No | Ex | II |
| **X131** | IHD | Yes | Yes | Ex | II |
| **X03** | IHD | Yes | Yes | Ex | III |
| **X105** | IHD | Yes | No | Ex | III |
| **X116** | IHD | No | Yes | Non | II |

To precondition cells, a final concentration of 100ng/ml SDF-1α (R&D Systems) was added to the tissue culture flask for either 1 hour (acute) or overnight (chronic). The bone marrow monouclear cells were in suspension, so therefore could only be preconditioned in an acute setting, otherwise their viability would have been compromised.

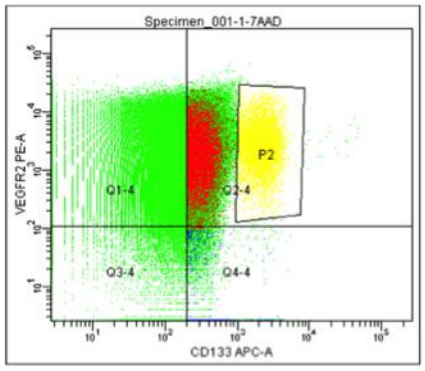
MNCs were obtained following written, informed consent and approval from the Local Research Ethics Committee in accordance with the Declaration of Helsinki, from patients recruited to the REGENERATE phase II clinical trial (NCT00747708). The REC responsible was South West REC and the reference number for the project is: 04/Q0603/13.

## Results

### Characterisation of bone marrow derived mononuclear cells

Isolated cells in the lymphocyte/monocyte region of the ficoll separation were characterised for their surface marker expression of CD34/CD45, CD133 and VEGFR2 (Figure 7B). The fractions of lymphocytes was also accounted for by CD45 positive cells with appropriate side scatter, with CD3 positive for T cells, CD19 positive for B cells, and CD15/56 positive for Natural Killer cells (Figure C).

A




B

| Surface marker | Cell population | Population Percentage (%) (±sd) |
|---|---|---|
| CD34+/45+ | Haemopoetic stem cells | 18.1 (±1.6) |
| High CD133/VEGFR2 | Endothelial progenitor | 1.5 (±0.04) |

C

| Cell | Population Percentage (%) |
|---|---|
| Lymphocytes | 30.5 |
| - T cells | 70.1 |
| - NK cells | 7.4 |
| - B cells | 11.3 |

Figure 7 Flow cytometry analysis of mononuclear cell isolations.

(A) Flow cytometry dot plot, of CD133 (APC) and VEGFR2 (PE). Highly expressing CD133+/VEGFR2+ cells appear in yellow, in box P2. (B) Population percentage of HSC and Endothelial progenitor cells according to surface markers CD35/45 and CD133/VEGFR2. (C) Lymphocyte constituents of mononuclear cell isolation

**Isolation of Bone Marrow derived Mesenchymal Stem cells**

Lymphocyte and monocytes cells, containing the stem cell population, were isolated by ficoll density centrifugation. Cells of mesenchymal morphology appeared after 4 days, and became confluent after 14 days (Figure 8). Mesenchymal cells appear at Day 4 (D4). D5-7 mesenchymal colonies can be seen. By D14 plate is confluent, containing mesenchymal stem cells and other unidentified adherent populations

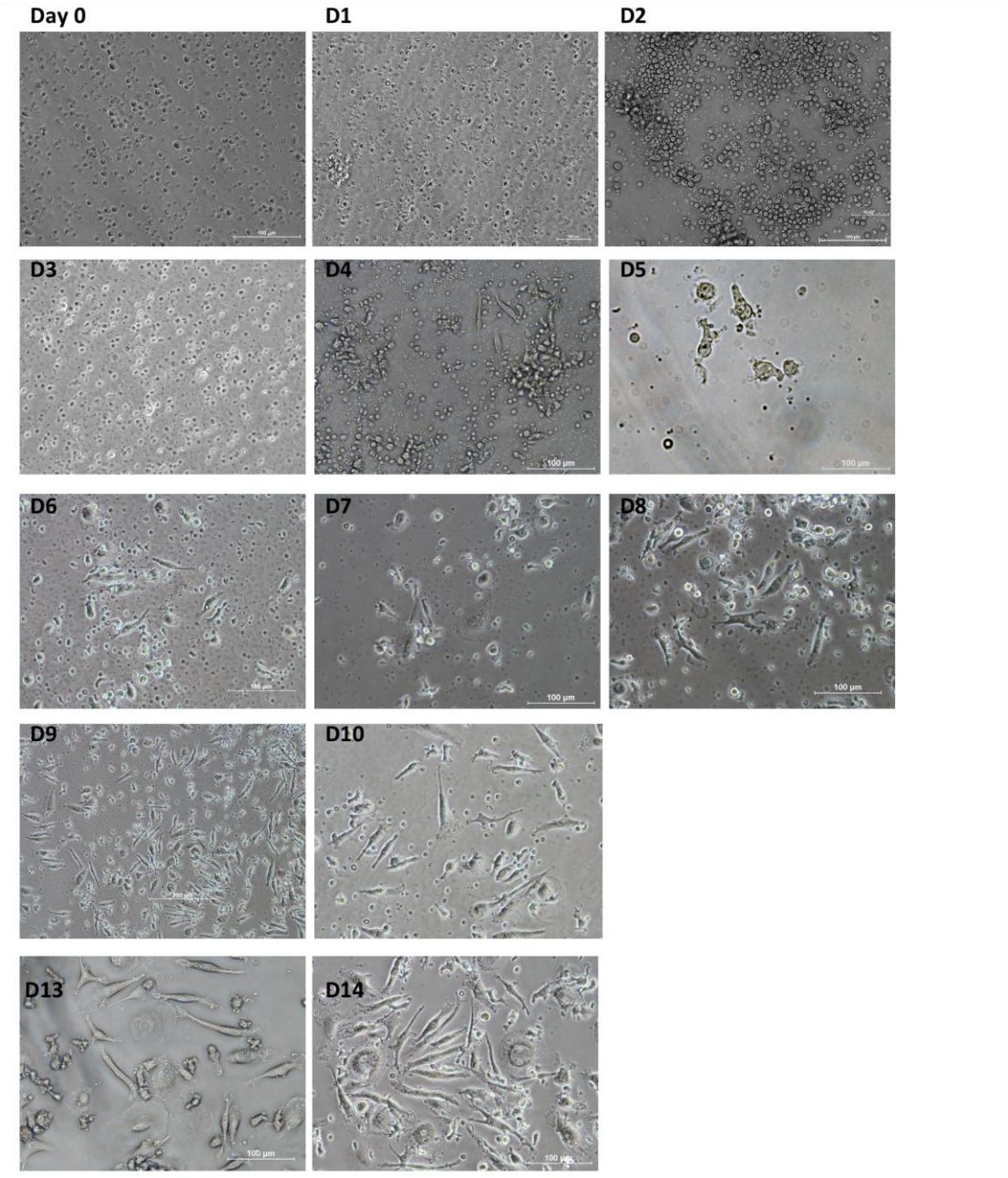



Figure 8 Chronological observation of mesenchymal stem cell growth from bone marrow isolation. Mesenchymal cells appear at Day 4 (D4). D5-7 mesenchymal colonies can be seen. By D14 plate is confluent, containing mesenchymal stem cells and other unidentified adherent populations

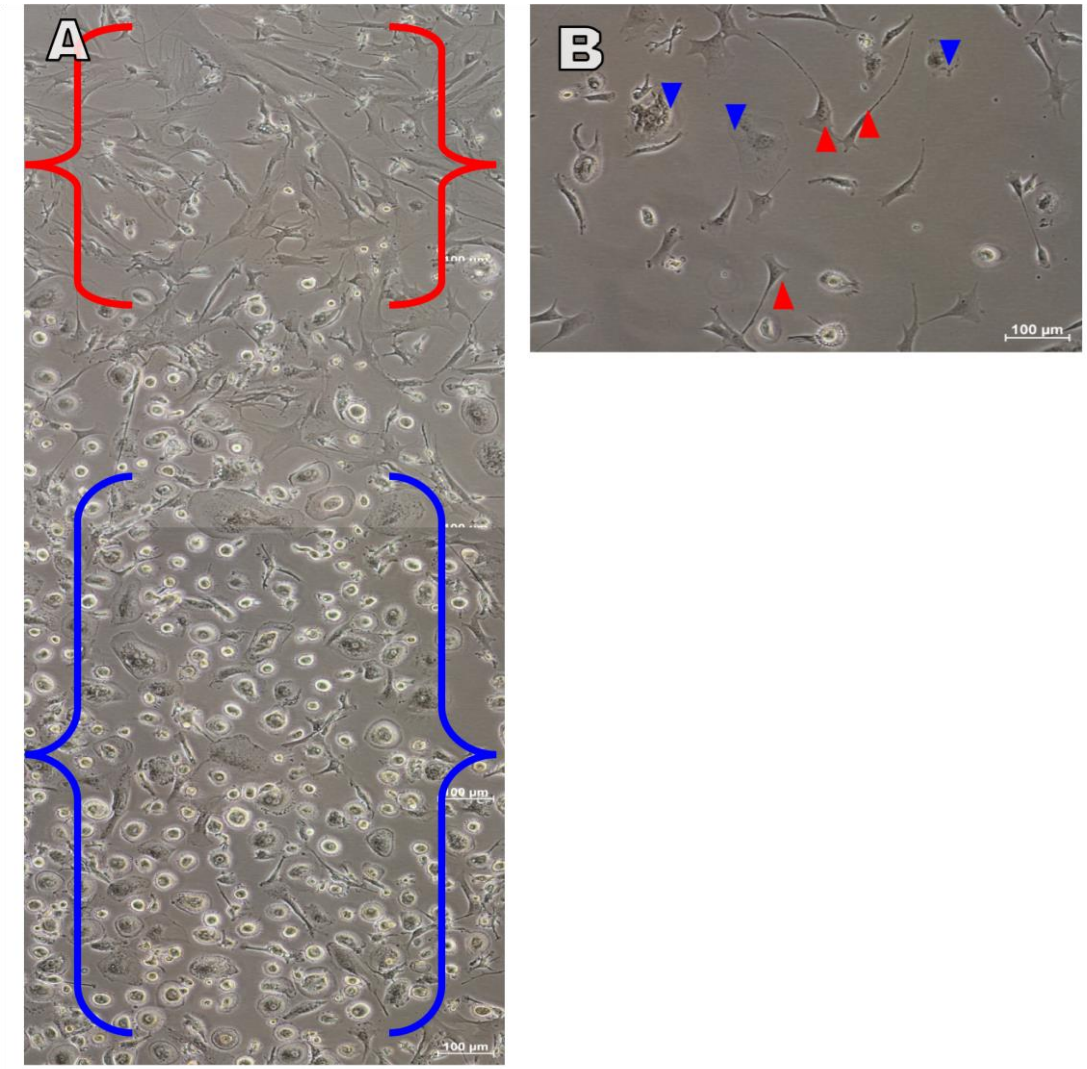



Figure 9 - Heterogeneous adherent cell population in the bone marrow

After density centrifugation of bone marrow sample lymphocyte and monocyte cells were cultured on plastic and after 12 days with regular changes the primary culture contained mesenchymal stem cells (according to morphology) and unidentified round cells, showing the bone marrow contains other adherent population of cells than MSCs. In figure A the round cells (BLUE region) are cluster on the tissue culture flask surround by MSC (RED region). Figure B shows a different area of same tissue culture flask that is less confluent containing cells of different morphology (red and blue arrows).

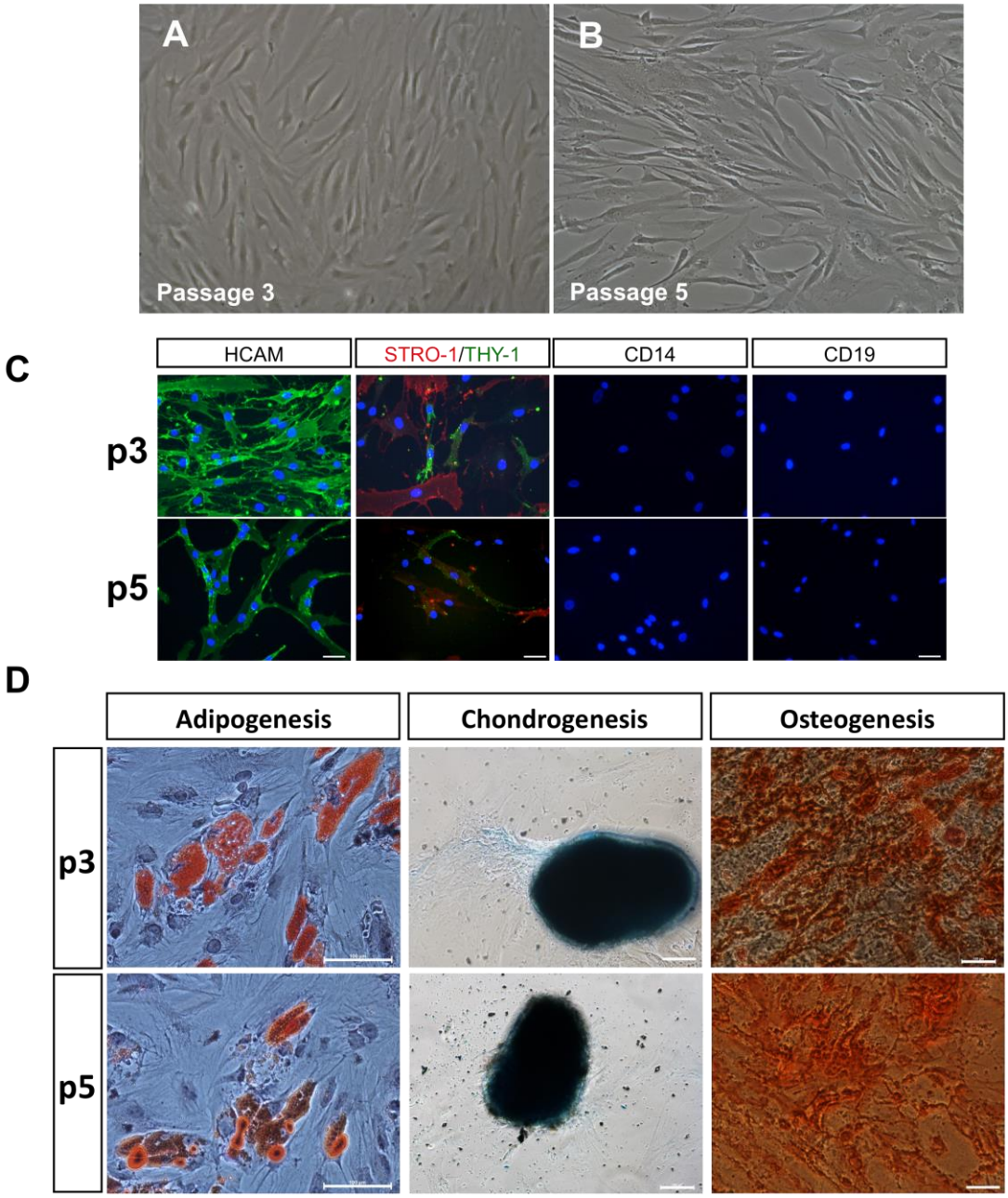



Figure 10 Characterisation of bone marrow derived MSCs. MSCs at passage 3 and 5 were characterised under the definition of the International Society of Cellular therapy. Cells are adherent to plastic and show a spindle like morphology (A-B). Black arrows represent cells that have become hypotrophic in passage 5. They were positive for HCAM, STRO-1/THY1 and negative for CD14 and CD19 (C). All MSCs displayed their plasticity to differentiate to adiopgenic, chondrogenic and

osteogenic lineages. MSCs were stained with oil red O, alcian blue and alizarin red respectively (D). Scale bars represent 100µm

MSCs were grown in DMEM with 10% FBS and 1ng/ml bFGF and cultured to 5 passages, and characterised at passage 3 and 5 in accordance to the ISCT guidelines. MSCs at passage 3 and 5 showed comparable morphology (Figure 10A-B), of a long and thin striated appearance with a large nucleus (Figure 10B). However in passage 5 some cells within the culture became enlarged and hypertrophic. Subsequent passages were observed to contain many cells with this morphology (data not shown). MSCs at both passage 3 and 5 expressed CD44, STR0-1 and THY-1 but not lymphocyte and T-cell markers CD14 and CD19 (Figure 10C). Both passages were capable of tri-lineage differentiation confirming identification as Mesenchymal Stromal Cells (Figure 10D).

**Adhesion of MSCs enhanced by fibronectin not SDF**

To determine the functional MSC attachment to ischemic myocardium I studied the effect of in vitro of SDF-1 preconditioning in time dependant adhesion to fibronectin, an extracellular matrix component upregulated after myocardial infarction. After expansion, MSCs were grown to 80% confluency, harvested and resuspended in serum free media. $1x10^4$MSCs were seeded onto BSA or Fibronectin (FN) coated wells in a 96 well plate. Significantly more cells adhered to fibronectin compared to wells coated with BSA, within 40 minutes for both passages (Figure 11B). Significantly more cells adhered to fibronectin over 20 minutes and this

was consistent in 2 different isolations. For MSCs in passage 3, neither acute nor overnight preconditioning with SDF-1 increased amount of cells adhere (Figure 12B). However, in passage 5, there was significantly more cells adherent than control cells on fibronectin ($p<0.05$) within 5 minutes (Figure12B)

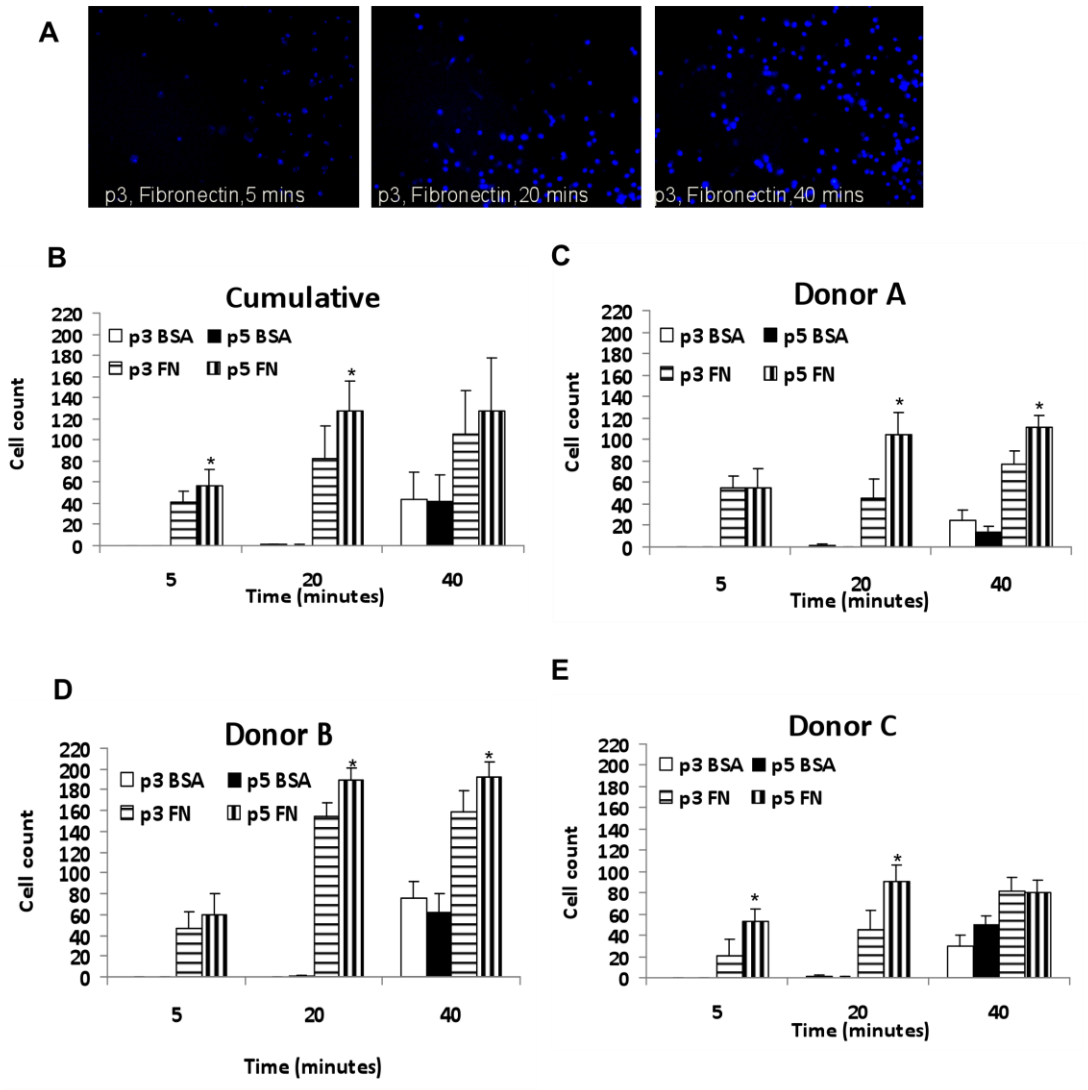
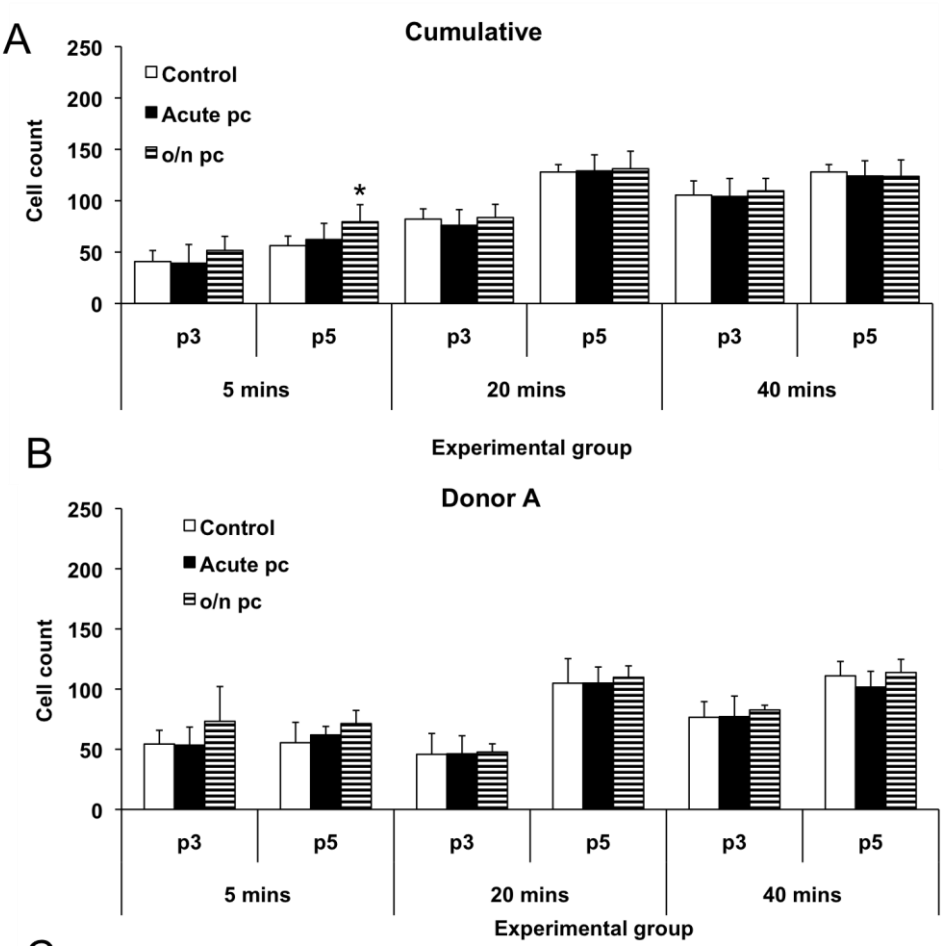



Figure 11 – Adhesion of MSCs. Passage 3 (p3) and 5 (p3) MSCs were grown in normal media to confluency and harvested. After resuspension, $1x10^4$ MSCs were seeded to BSA and fibronectin for 5,20 and 40 minutes, fixed and stained for DAPI for counting on ImageJ software (A). MSCs adhere preferentially to fibronectin than non-specific protein in 3 different MSC isolations (B-E). *=$p<0.05$ between p3 and p5


A
Cumulative
Control
Acute pc
o/n pc
Cell count
250
200
150
100
50
0
*
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p5
p3
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p5
5 mins
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40 mins
Experimental group
B
Donor A
Control
Acute pc
o/n pc
Cell count
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p5
5 mins
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40 mins
Experimental group

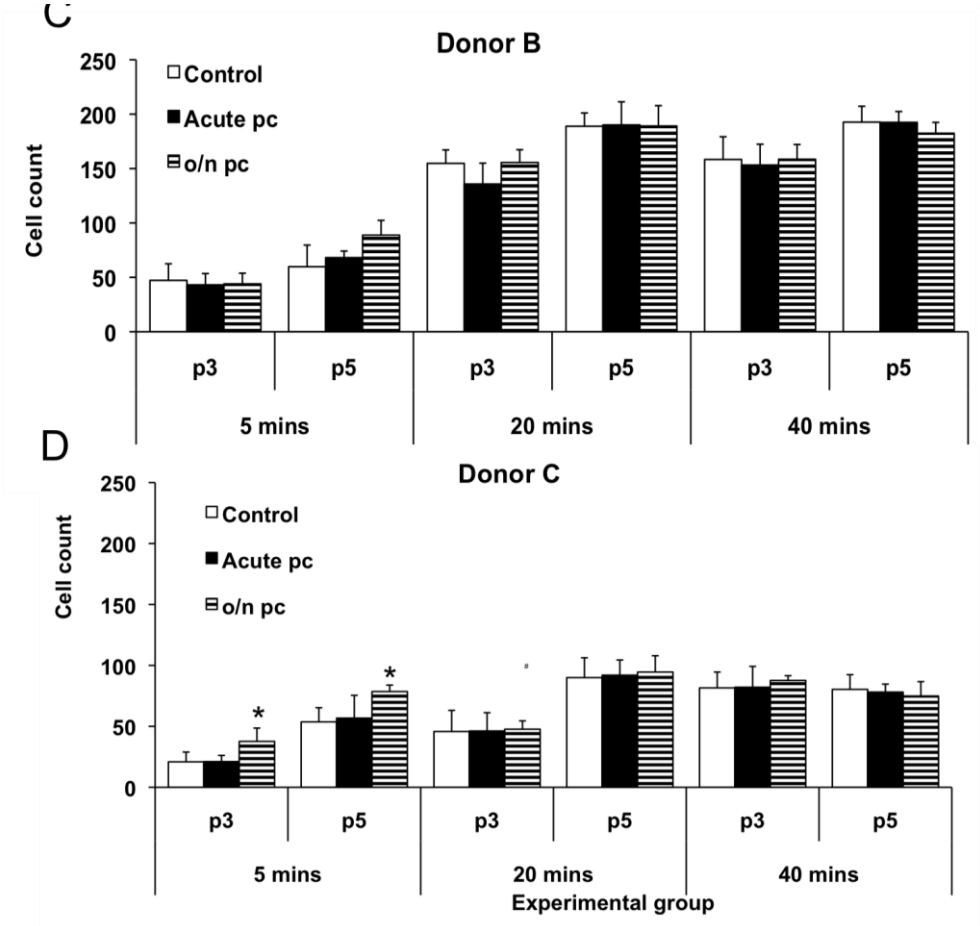



Figure 12 – Effect of SDF-1 conditioning for MSC adhesion. MSCs were stimulated with SDF-1 either for 1 hour (acute pc) or overnight (o/n pc). SDF-1 had increased adhesion of passage 5 cells over minutes (A) and in isolations from Donors B and C (C-D). *=p<0.05 versus control in time course

Adhesion of BM-MNC increased by fibronectin, not SDF-1 preconditioning. Bone marrow mononuclear cells adhered significantly greater to Fibronectin than a non-extracellular matrix regardless of SDF-1 stimulation (Figure 13A-D). This is consistent for each patient sample that was used, however BM-MNCs from patient 31 (Figure 13D) had greater amount of cells adhered after 20 minutes for all experiment groups than cells from patient 131 (Figure

13C). This variability dependant on patient isolation is represented in the cumulative data (Figure 13A)

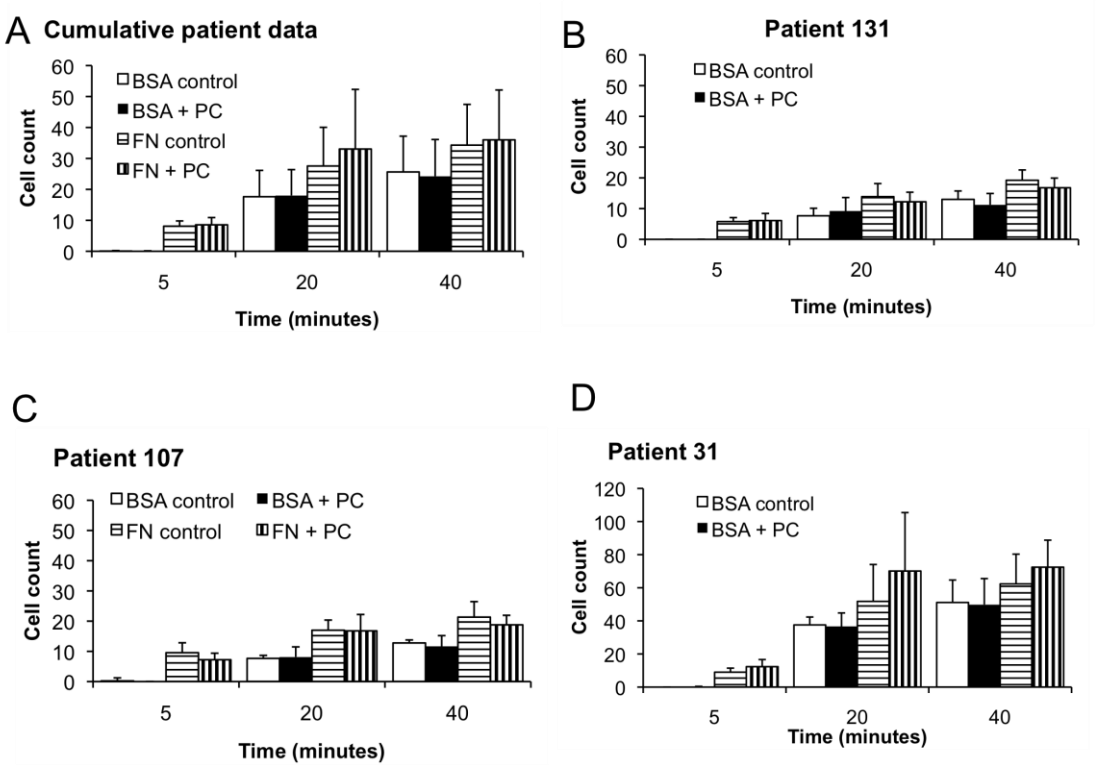



Figure 13 SDF-1 does not increase adhesion of BM-MNCs. BM-MNCs were obtained from individual patient samples from the REGENERATE – IHD and DCM clinical trial (A-C). BM-MNCs were preincubated in hMSC media in the absence or presence of SDF-1 (100ng/ml) for 30 mins (PC) and overnight and were allowed to adhere to the wells FN and BSA coated wells for 5, 20 and 40 mins (A). Adhesions were quantified by microscopy. Adhesion data represent the mean ±SD of triplicate samples from 3 independent experiments (B-D). BSA – Bovine Serum Album coated wells; PC- preconditioned cells (100ng/ml SDF-1) FN- Fibronectin coated wells. *= $P<0.05$ between BSA control vs. FN groups

### 3.3.1 MSCs migrate towards an SDF-1 gradient

SDF-1 is over expressed by the ischemic myocardium for 7 days (Penn, 2009), the chemotaxis of MSCs to SDF-1 was determined in a transwell

migration assay. I investigated whether preconditioning cells with SDF-1 would enhance the migration response. MSCs were grown in normal growth media and harvested at 80% confluency for use in the chemotaxis experiments

Both unstimulated and stimulated groups showed significant migration towards SDF-1 compared to control. Interestingly, while preconditioning of passage 3 MSCs with SDF-1 did not affect migratory response, passage 5 cells preconditioned with SDF-1 had a significant increase in cells ($p<0.05$). In addition passage 5 cells have diminished chemotaxis to SDF-1(Figure 14B). Migration was also variable between individual MSC isolations

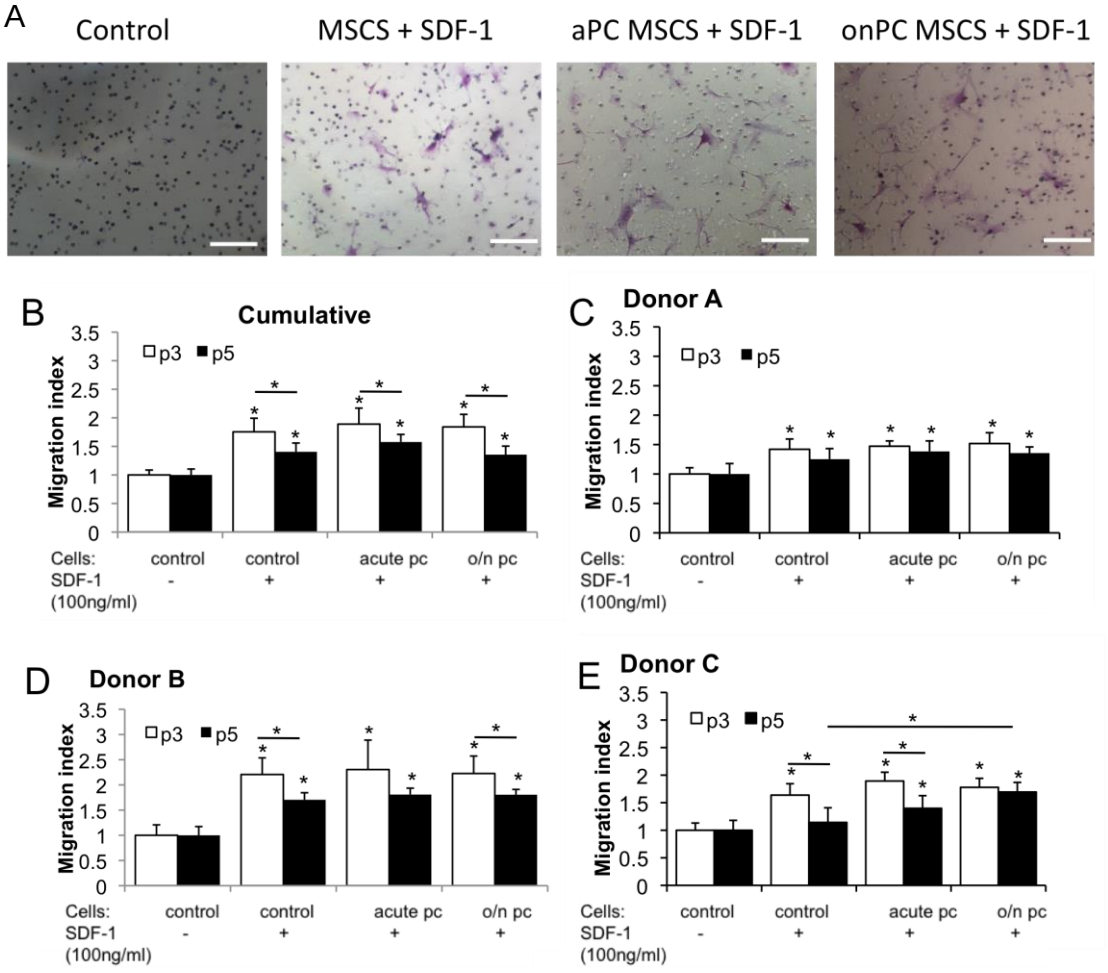


Figure 14 MSCs preferentially migrate towards SDF-1. The effects of MSC migration were determined by exposing MSCs to 100ng/ml SDF-1 in the lower chamber of the transwell for 3 hours. The transwell membrane was not coated. Cells were placed in the transwell upper chamber with serum free media. After 3 hours of incubation non-migrating cells were scraped off with cotton swabs. Migrated cells on the membrane were stained with crystal violet, images taken at x10 magnification (A). The migrated cells were quantified by observing three random microscopic fields for each membrane (B). Control – MSCs in serum free media and serum free media in lower well. Both unstimulated and stimulated groups have SDF-1 (100ng/ml) in lower well. This was repeated using 3 different MSC isolations (C-E) * = $p<0.05$

**BM-MNC chemotactic response to SDF-1 is population specific**

The chemotactic response of BM-MNC to SDF-1 was investigated in a transwell migration assay. 3 patient BM-MNC samples were used and chemotactic response to SDF-1 was varied (Figure 15). Isolate 31 showed a 10-fold increase compared to the control, while x131 displayed no increase when compared to control.

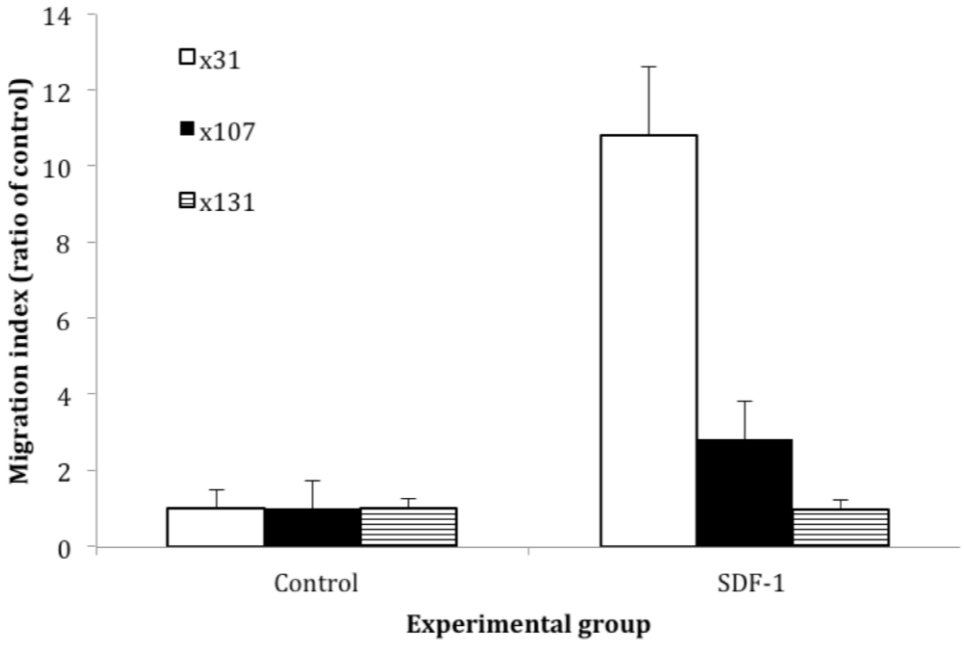



Figure 15 - Chemotaxis of BM-MNCs to SDF-1. Cells were seeded into a transwell with 8um pores with and without SDF-1 in the lower well as the chemoattractant .x31, x107, x131 represent individual patient samples of BMMNCs. *=p<0.05 versus control

**SDF-1 stimulation of MNCs stimulates EPC phenotype**

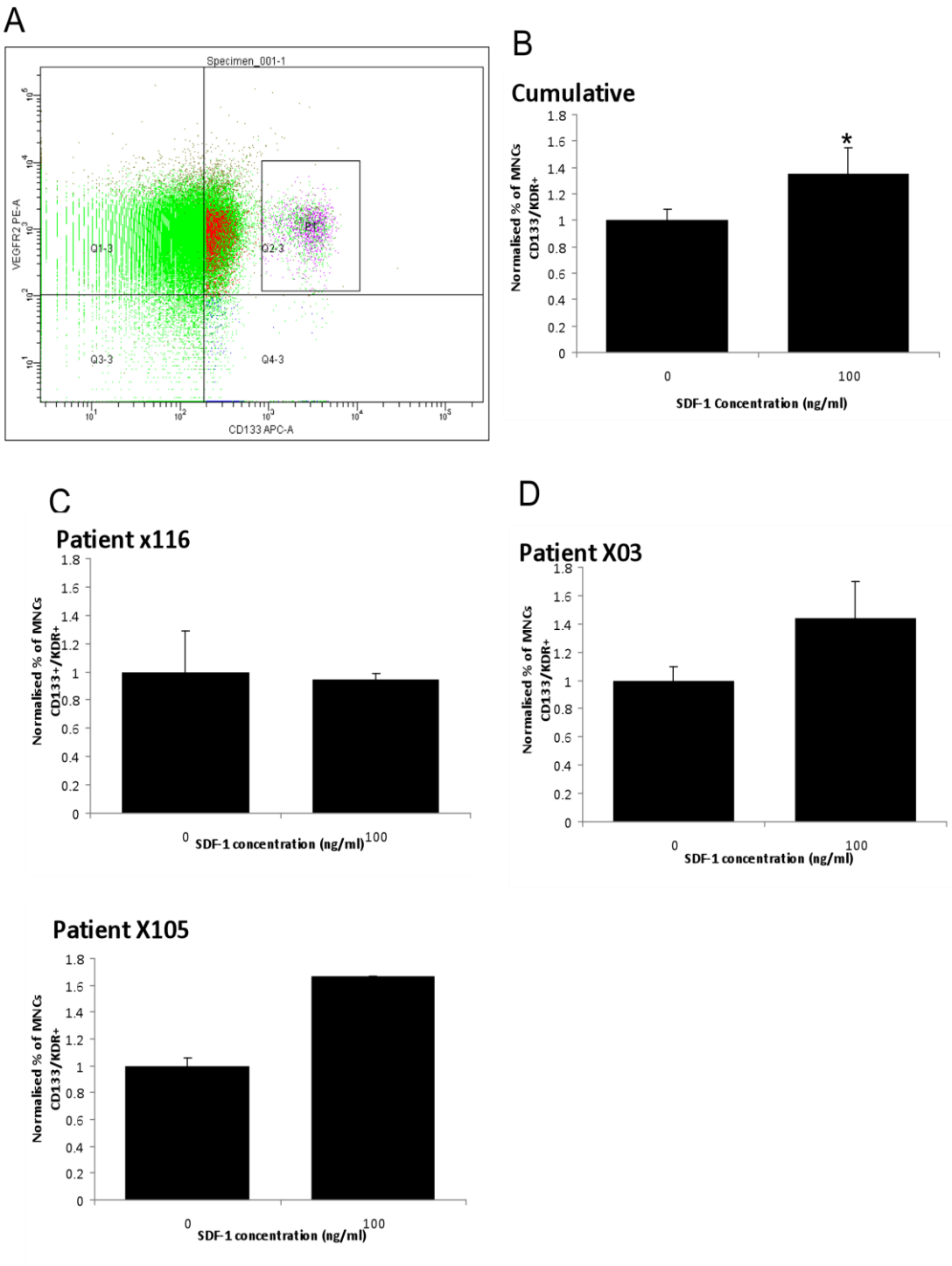
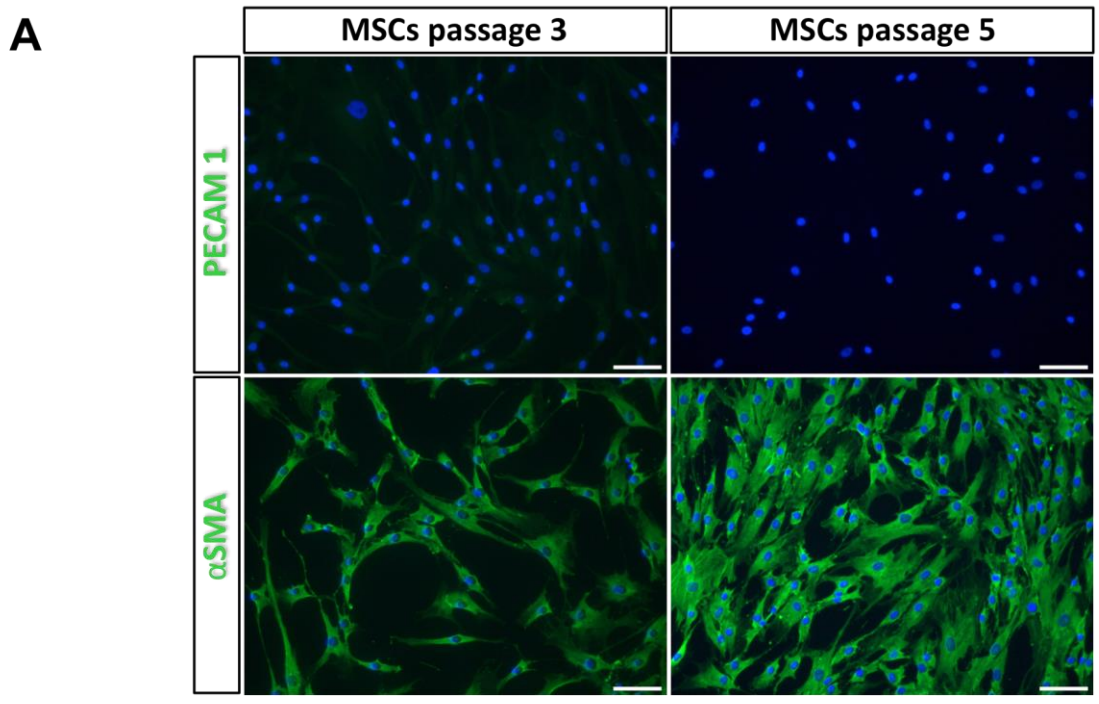
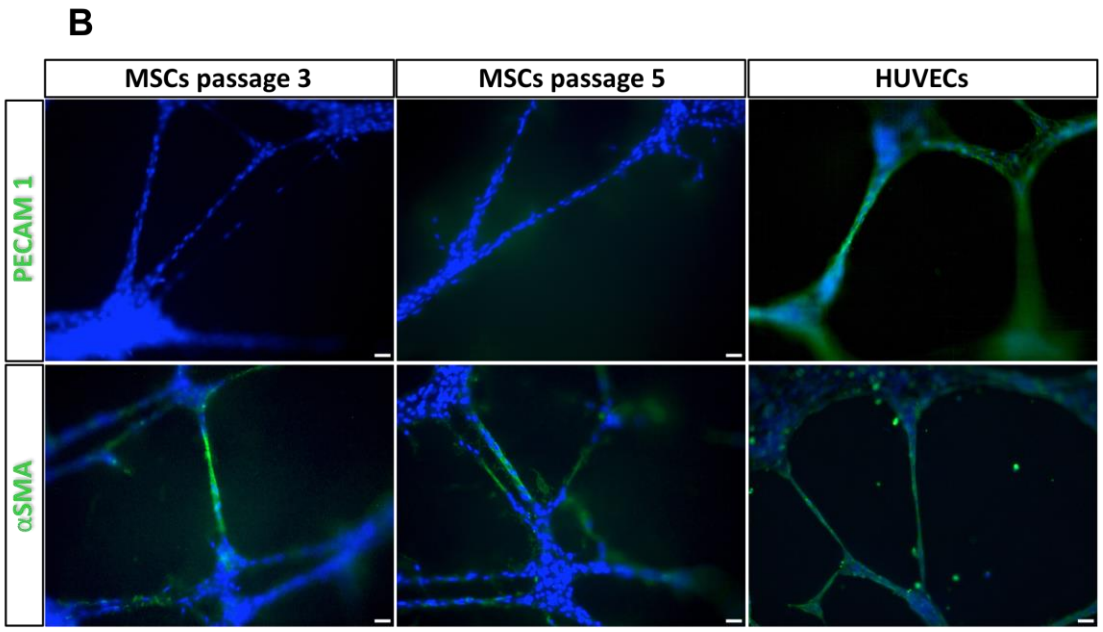


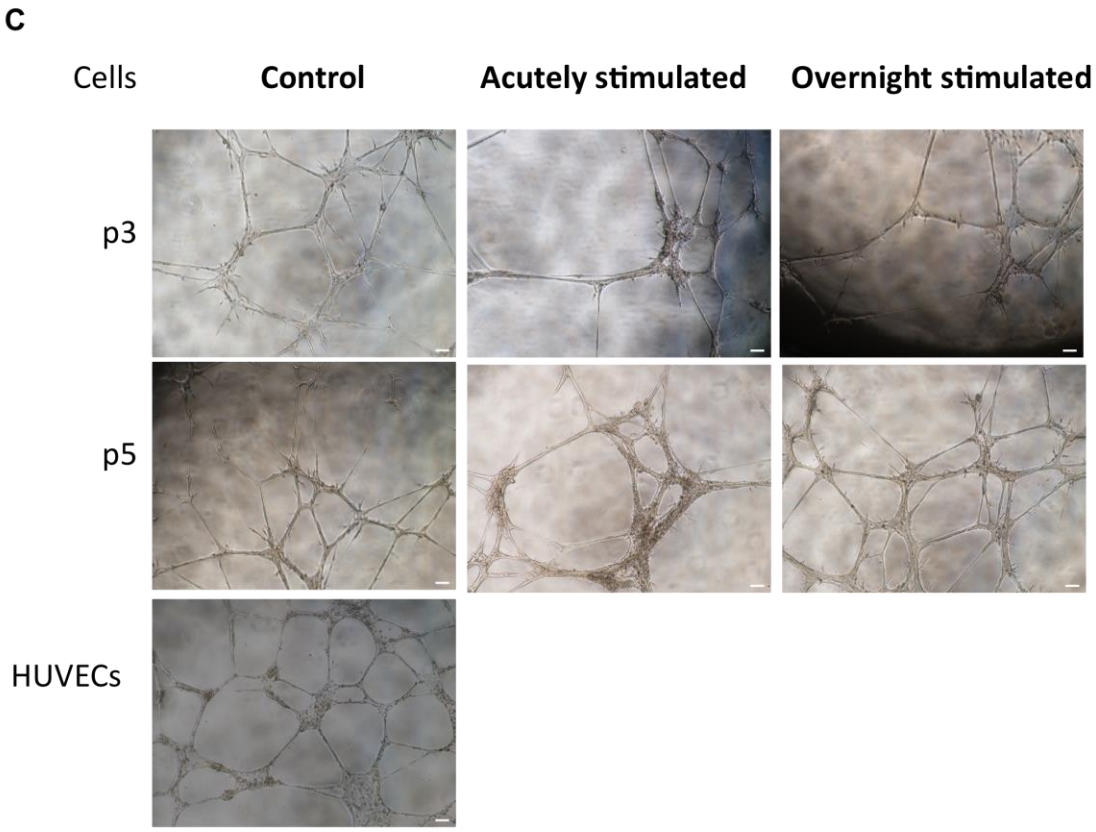

Figure 16 Flow cytometry analysis of patient derived MNCs. A population of MNCs highly expressing CD133 and VEGFR2 (KDR) was gated (A). MNCs were stimulated with control or SDF-1 (100ng/ml) for 30 minutes. Then cells were analysed by flow cytometry for CD133/KDR expression (B). This was repeated with 3 different patient isolations (C-E)

**SDF-1 does not increase in vitro tubule like structures formation of MSCs**

To investigate the network forming capacity, MSCS were seeded onto matrigel in EGM-2. EGM-2 contains several growth factor and supplements that include epidermal growth factor, insulin like growth factor 1, VEGF, ascorbic acid, hydrocortisone, bFGF when seeded onto matrigel with EGM-2 media have a distinctive phenotype change to form tubule like structures (Figure 17A), however no structures were formed when seeded with normal culture media., These structures were quantified for branch point formation (Figure 17B) and no difference was found in those cells preconditioned with SDF-1. HUVECs formed more uniform vessel structures and had 1.6 fold more branch formations. MSCs are alpha smooth muscle actin (αSMA) expressing and Platelet/endothelial cell adhesion molecule 1 (PECAM-1) negative, while HUVECs are PECAM-1 expressing, and weakly expressing αSMA




B
MSCs passage 3
MSCs passage 5
HUVECs
PECAM 1
αSMA


C
Cells
Control
Acutely stimulated
Overnight stimulated
p3
p5
HUVECs

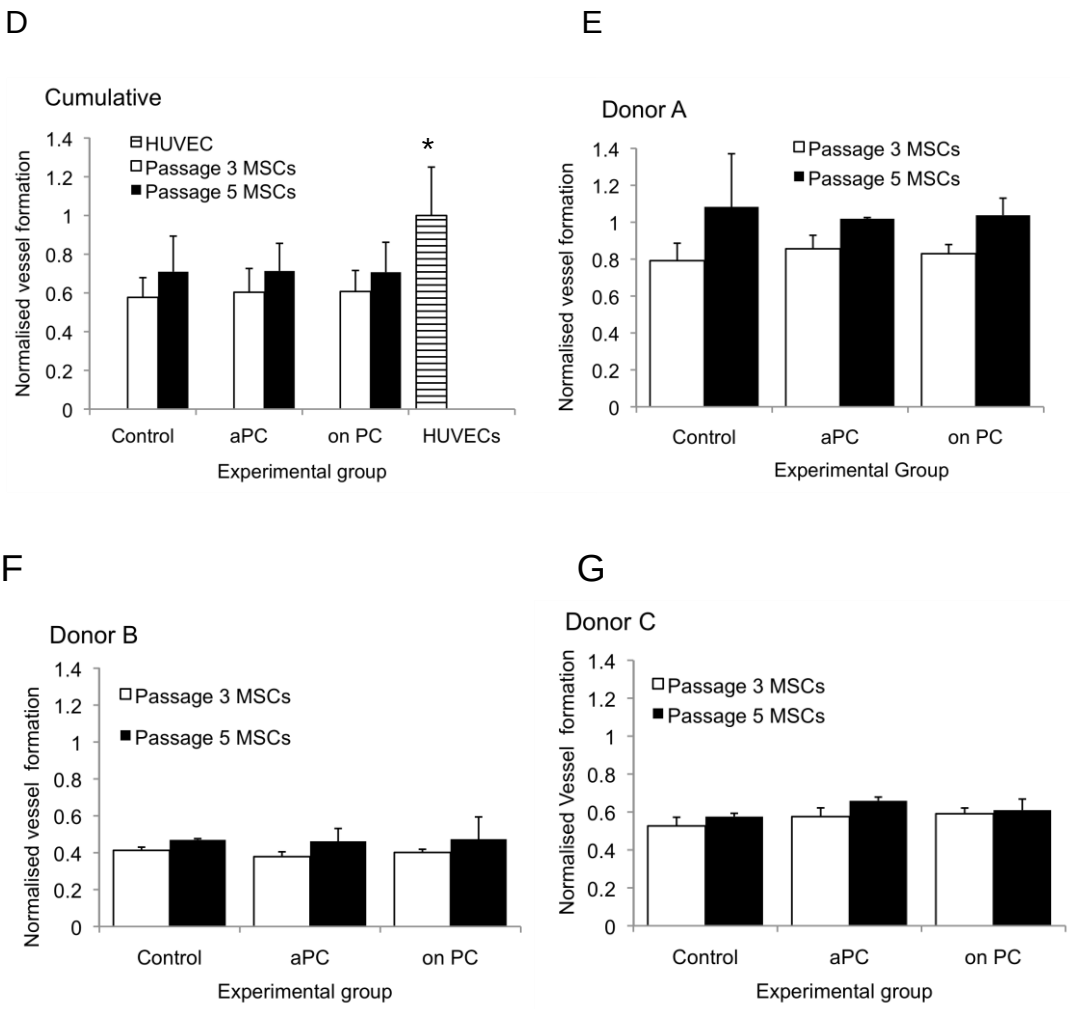



**Figure 17 - SDF-1 does not increase tubule like structures.** Cells were fixed and expression of PECAM-1 and aSMA and DAPI (for nuclei staining) detected by immunocytochemistry in matrigel (A) and tissue culture plastic (B). Images taken at x10 magnification. MSCs were added to 96 well plates coated with matrigel with and without SDF-1. HUVECs were used as a positive control. Plates were incubated for 18hours to allow tubule like structure formation (C) Individual vessel formations were quantified (D-G) * = $p<0.01$ vs control. Scale bars represent 100um

**BM-MNCs do not form tubule like structures in Matrigel**

BM-MNCs from patient samples were seeded onto matrigel and after 18 hours, unlike the MSCs, did not form tubule like structures, and clustered in the centre of the well (Figure 18A). This was not affected by SDF-1 preconditioning (Figure 18B)

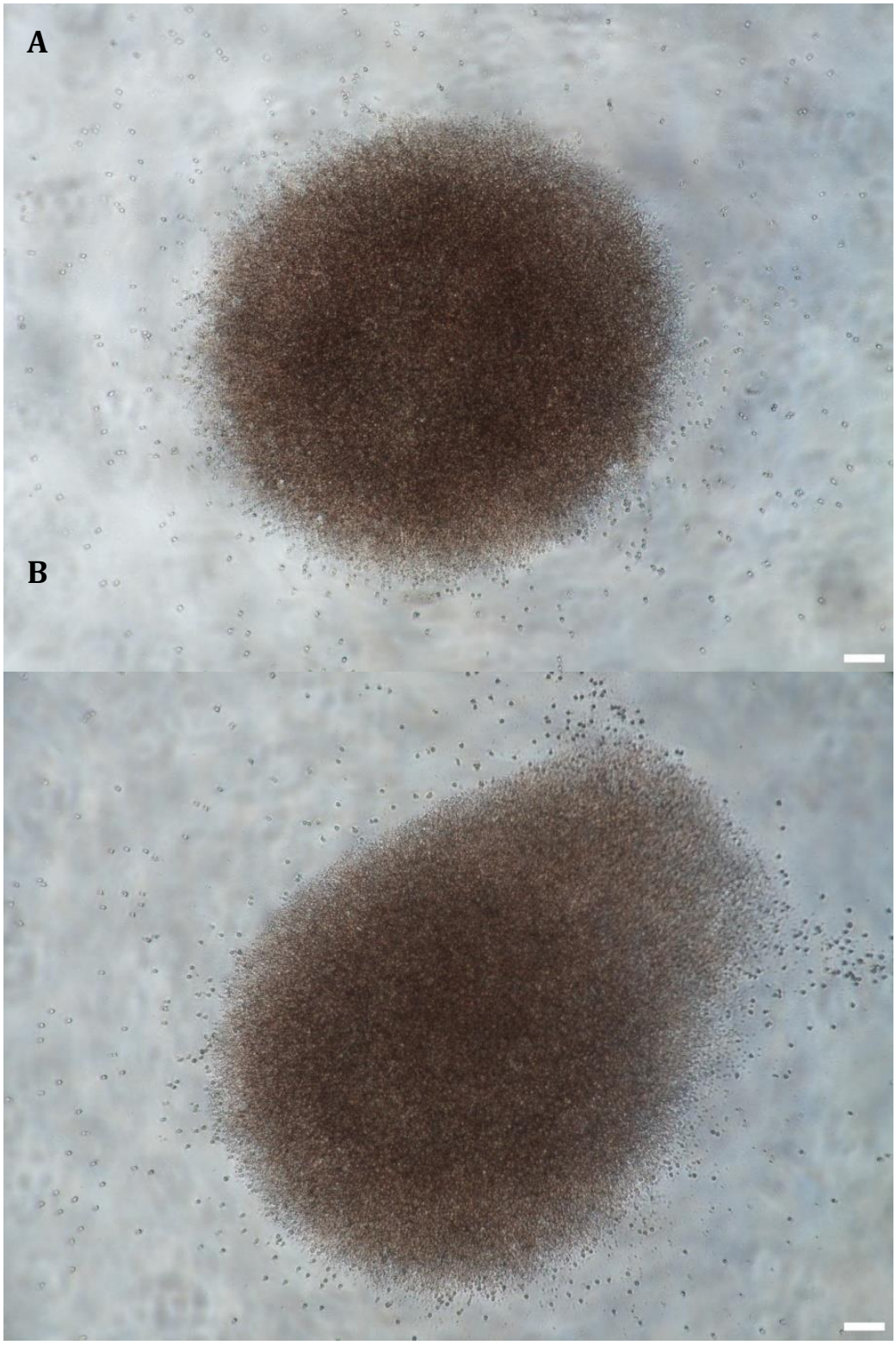



Figure 18 - MNCs do not form tubule like structures in matrigel. MNCs did not form any tubular network (H) regardless of preconditioning (I). Scale bars represent 100um

**Hypoxia abrogates cell adhesion**

In order to replicate the ischemic environment, the assays were repeated in a hypoxic chamber purged with 2% oxygen. Significantly less cells adhered to fibronectin within 5 and 20 minutes in the hypoxic environment compared to normoxia (Figure 19). This trend was repeated with the patient MNCs and consistent in all isolations (Figure 20). However, no effect was observed in migration or tubule formation of the MSCs in hypoxia compared to normoxia (Figure 21). However, donor A showed significant decrease in migration, and increase in number of vessel branches formed in hypoxia compared to normoxia. Hypoxia did significantly increase vessel formation in one isolation, but no affect in the other 2 (Figure 22B).

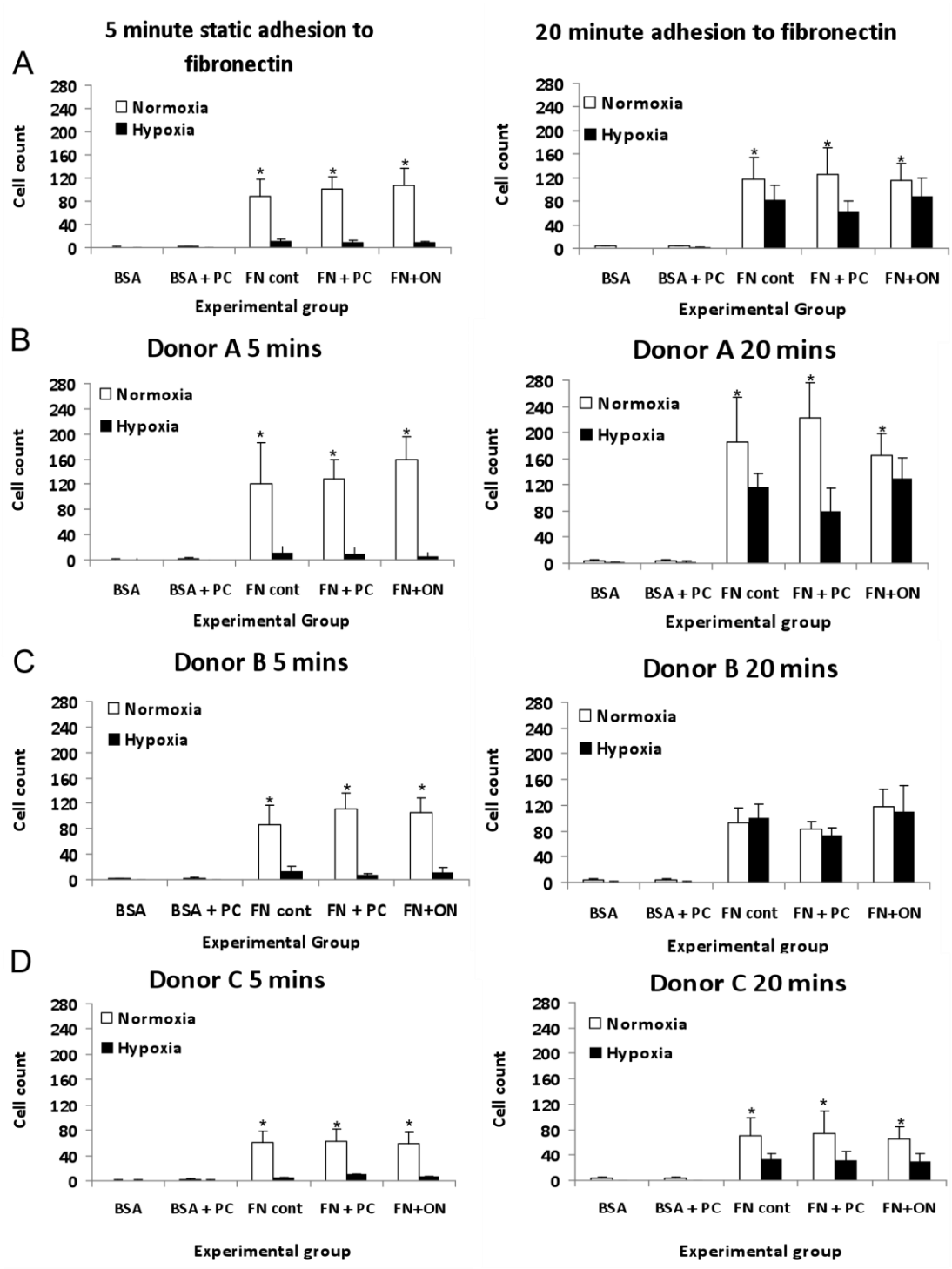



Figure 19 Effect of hypoxia on MSC attachment. The effect of hypoxia was assessed in passage 3 MSCs for 5 minutes (A-D) and 20 minutes (E-H). Hypoxia impaired rate of attachment to FN. Preconditioning with SDF1 did not affect attachment. Data represents mean ± SEM for (A, E) and mean ± SD for each independent experiment (B-D, F-H). * = $p<0.05$ Normoxia vs hypoxia.

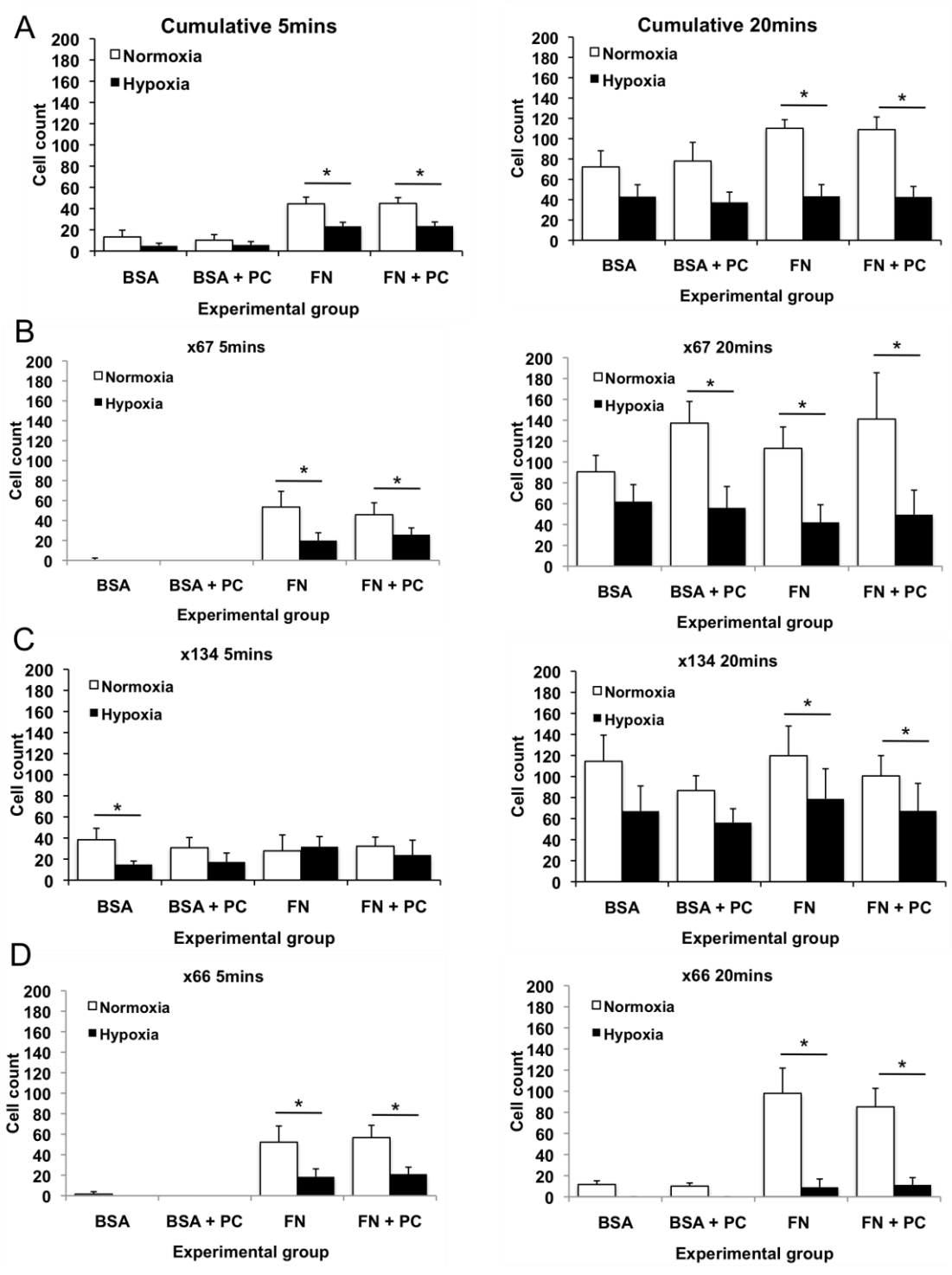



Figure 20 Effect of hypoxia on MNC attachment. The effect of hypoxia was assessed in passage 3 MSCs for 5 minutes and 20 minutes. Hypoxia impaired rate of attachment to FN. Preconditioning with SDF1 did not affect attachment. Data represents mean ± SEM for (A) and mean ± SD for each independent experiment (B-D). * = $p<0.05$ Normoxia vs hypoxia.

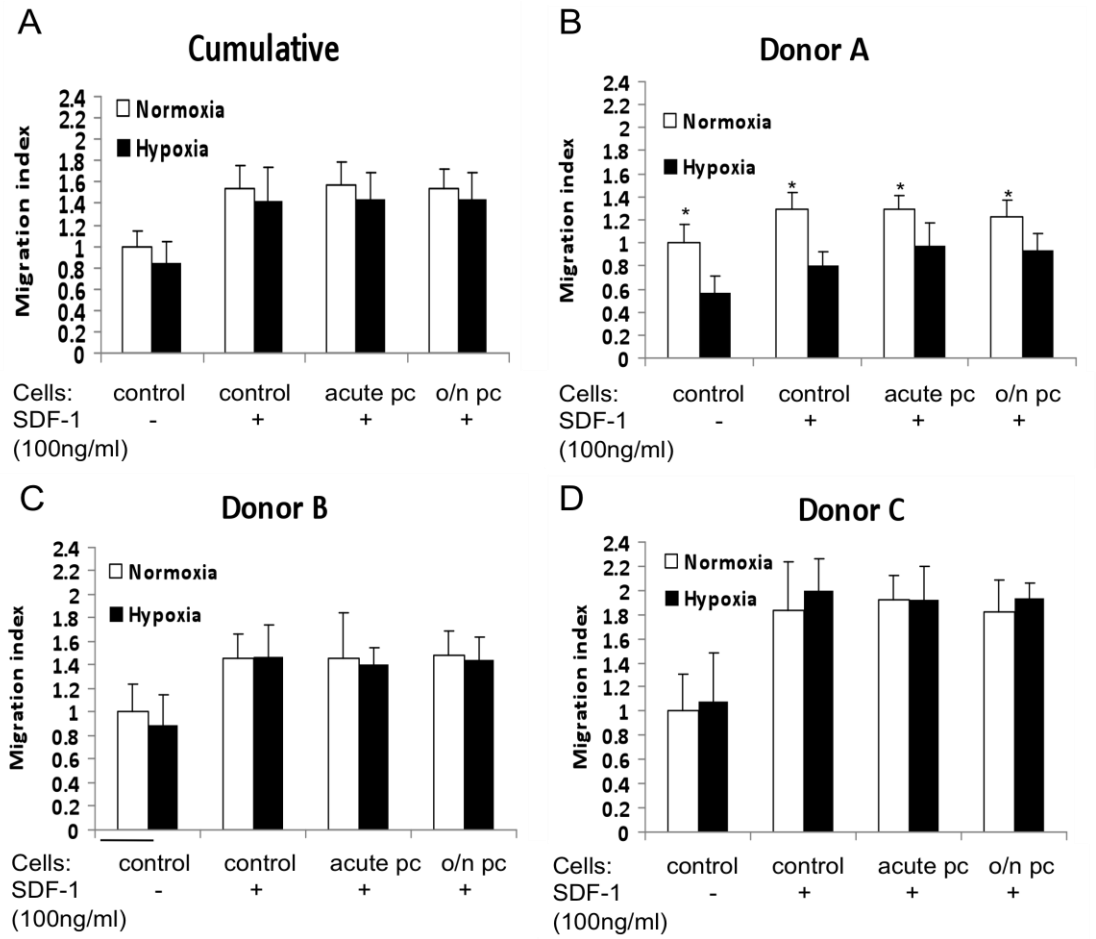



Figure 21 Effect of hypoxia on MSC chemotaxis. Chemotaxis of passage 3 MSCs overall appeared not affected by hypoxia when combined data was assessed (A). However, when considering individual donor samples (B-D), evidence of impaired migration was seen in MSCs from one of the three independent donor samples (B). Data represents mean ± SEM for (A) and mean ± SD for each independent experiment (B-D). * = $p<0.05$ Normoxia vs hypoxia.

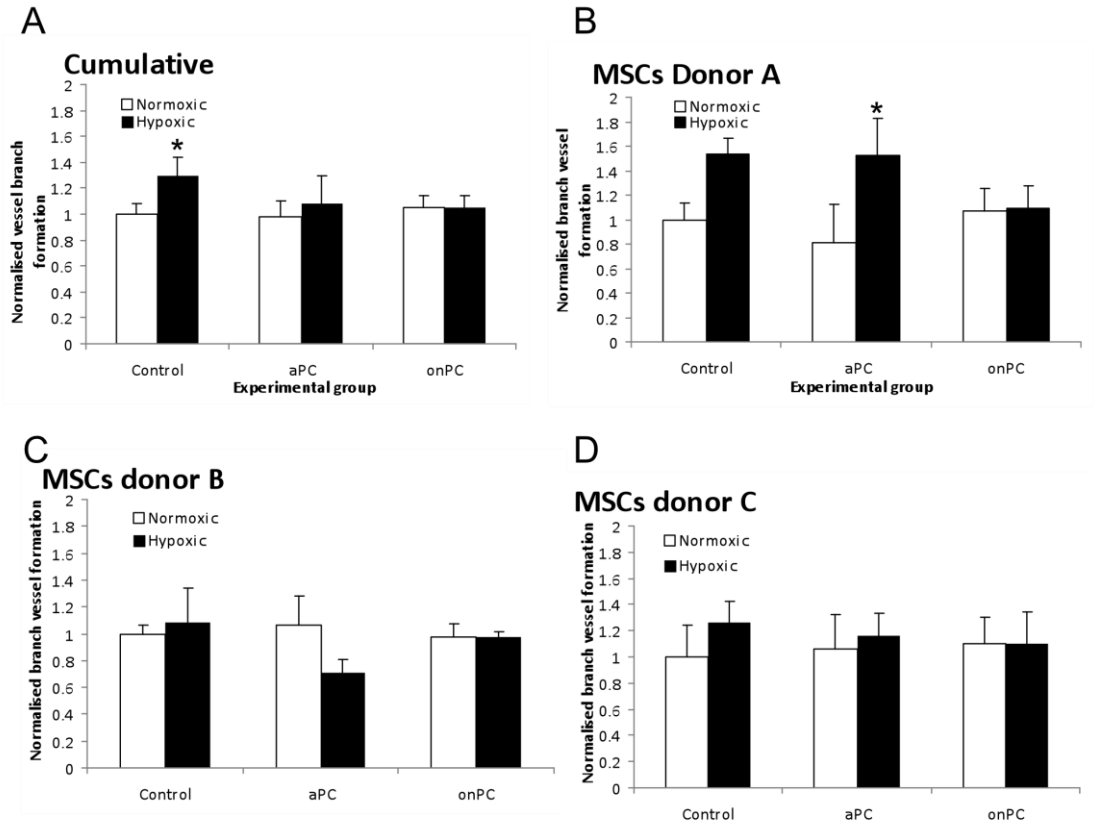



Figure 22 - Effect of hypoxia on MSC tubule formation. Hypoxia had some significant increase in tubule formation on the control MSC group (A), but not for preconditioned groups. Data represents mean ± SEM for (A) and mean ± SD for each independent experiment (B-D). * = $p<0.05$ Normoxia vs hypoxia.

## Discussion

SDF-1 is a chemokine that is implicated in migration of BM-MSC and Haematopoietic stem cells (HSCs). The chemokine is over expressed in the ischemic myocardium for 7 days post infarct (Ghadge et al., 2008). SDF-1 is a chemoattractant to cells expressing its receptor CXCR4 (Kucia et al., 2004). Preclinical studies in rat myocardial infarction (MI) model have shown that preconditioning of MSCs with SDF-1 can improve their survival, can increase vasculature in the ischemic myocardium, and decreased infarct size (Pasha et al., 2008). However, the effect to engraftment and chemotaxis was not studied. In addition, these findings may be as a consequence of the increased cell survival. Therefore this study aimed to show the in-vitro role of SDF-1 preconditioning in MSCs and BM-MNC for adherence, migration and vessel formation.

The major findings of this study found that: i) the extracellular matrix component, Fibronectin, was more important for to adhesion than SDF-1 preconditioning; ii) MSCs can form similar tubule like structure in a matrix to HUVECs, but is not enhanced by preconditioning; iii) BM-MNCs do not form tubule like structures; iv) MSCs and BM-MNCs migrate towards a SDF-1 gradient; v) Migration response of MSCs is dependent on their maturity, and the response of passage 5 cells is enhanced by SDF-1 preconditioning.

Post-MI several extracellular matrix components are upregulated, including fibronectin (Knowlton et al., 1992). SDF-1 has been to shown to upregualte $\alpha 5\beta 1$ in another cell type present in the bone marrow,

endothelial progenitor cells (Ghadge et al.). This integrin is vital for firm adhesion to fibronectin therefore upregulation would enhance amount of cells adhered. The majority of animal studies that have shown a greater number resided within the myocardium results in increased cardiac function

Passage 5 MSCs stimulated overnight with SDF-1 adhered to fibronectin significantly greater in 5 minutes compared to unstimulated MSCs. This was abrogated after 20 minutes where there is no difference. There is no difference between adhesion at 20 and 40 minutes for all experimental groups, so most adhesion occurs within 20 minutes. Rapid firm adhesion is vital within the ischemic myocardium as many cells are lost to other parts of the body. Suzuki *et al* showed that that 44% of transplanted cells survived for 10 minutes and decreased to 15% within 24 hours (Ohtsuka et al., 2004). Using a hypoxic culture chamber, we characterized rapid attachment of hMSCs up to 20 minutes and found an enormous significant reduction in hypoxic conditions. Hypoxia has also been reported to reduce attachment of endothelial cells (Kaiser et al., 2012) However, it is of particular significance for transplantation of hMSCs into ischemically injured tissues. Furthermore, preconditioning cells with SDF1 was not able to prime cells to rescue this phenotype.

The ischemic myocardium releases SDF-1 into the microenvironment and recruits bone marrow mesenchymal cells (Abbott et al., 2004; Zhuang et al., 2009). This study shows MSCs preferentially migrate towards SDF-1. Chemotaxis occurs via the SDF-1/CXCR4 axis so changes to the CXCR4 expression will affect migration (Cheng et al.,

2008; Ip et al., 2007). After multiple passaging of MSC ex-vivo culture CXCR4 expression is markedly reduced (Fiedler et al., 2004; Wynn et al., 2004). Passage 3 and 5 are significantly different in their migratory response due to reduced CXCR4 expression. The importance of CXCR4 expression is reinforced by a study that identifies MSCs exogenously over expressing CXCR4 expression have a three fold increase in migration to SDF-1 than non transfected MSCs (Cheng et al., 2008). MSCs at passage 5 have diminished chemotactic response that is increased with preconditioning with SDF-1. SDF-1 has been shown to induce an autocrine loop whereby CXCR4 is upregulated (Ghadge et al., 2008), thereby enhancing chemotaxis. However, this enhancement does not increase it that of the lower passage. This is important for therapy, as homing towards the infarcted myocardium may be reduced by low CXCR4 expression, therefore use of MSCs at a lower passage would be recommended.

There was no trend between patient derived BM-MNC chemotaxis to SDF-1. The response varied from a 10 fold increase to no change when compared to control. Therefore, this individualised response would dramatically impact the effect these cells would have when transplanted into the ischemic microenvironment. Isolated Bone marrow mononuclear cells contain a heterogeneous population of cells and have been the subject of clinical trials in cardiac repair for the past decade. This variability between samples could induce variable end outcomes. No statistical conclusions can be made between the assay outcomes and the medical history of the patients from which the cells were obtained.

However, cells from ex smokers (X107 and X131) adhered and migrated less than those from a non smoker (X31). MNCs contain a small population of EPCs. Another study have been shown EPCs to become dysfunctional and senescent in smokers, the same phenomenon could be occurring in the less responsive patient cells, although this is purely speculative (Michaud et al., 2006).

While the mechanism of action is still unclear for MSC therapy, animal models have previously shown preconditioning with SDF-1 enhances capillary density within the ischemic myocardium (Pasha et al., 2008). I mimicked in an *in vitro* 3D matrix assay that shows tubule formation, but SDF-1 does not enhance total branch formation. However, the limitations of the *in vitro* model do not account for any increased cell survival, engraftment or migration that may occur *in vivo* model. Also, the morphological features of the tubules bear little resemblance to capillaries formed *in vivo*. In addition the assays are carried out in EGM-2 that includes Vascular Endothelial Growth Factor and Insulin Growth Factor known angiogenesis stimulants. Their potency could mask any potential effect SDF-1 may have. In addition, BM-MNCs show no morphological changes in this assay. The heterogenous population of MNCs was not expected for these cells to form tubules on their own within matrigel due to a high proportion of haematopoeitc stem cells and macrophages that do not form this phenotype. However, their interaction with an existing vasculature is unknown.

It is notable that different isolations had variable results within the assays and response to SDF-1. If MSCs were to be used for therapy, using

different donors could therefore have differing outcomes. For instance those MSCs that show increased engraftment to fibronectin would be a lot more suitable. These assays could be utilised to identify suitable cell lines. Few studies have highlighted the disparity between isolations. Lo Surdo and Bauer showed differences of differentiation capacity and clonogenicity, albeit only comparing 2 different donor isolations (Lo Surdo and Bauer, 2012).

A possible source of variability is the FBS used in the normal growth media. FBS is produced by extraction of clotted whole blood collected from the fetus by cardiac puncture (Wappler et al., 2013). Therefore in each extraction there is lot to lot variability, as each batch contains differing amounts of proteins and other growth factors. Using different batches of FBS could have resulted in different growth characteristics and function of the MSCs.

The key findings from this study are that: i) By passage 5 using a standard cell expansion procedure, the attachment and chemotactic migration profiles of hMSCs was significantly altered and therefore the degree of subculture can impact on critical cell functions even where potency is seemingly conserved; ii) SDF-1 preconditioning did not substantially improve these responses other than a mild initial (5 minute) improvement in attachment indicating that sustained effects of SDF1 reported in some animal studies is independent of attachment and migration capacity of MSCs; iii) Hypoxia significantly impairs the rapid attachment of hMSCs to fibronectin but that migration is not significantly affected according to cross donor analysis; iv) Inter-patient variability is

substantial and therefore can make measurement of essential functional responses (and consequently development of assays to measure function and potency of the cell product) difficult.

## Conclusion

This study shows there is no evident effect of the engraftment by preconditioning bone marrow derived cells with SDF-1 either in an acute or chronic setting. The SDF1/CXCR4 axis is considered to be vital for myocardial repair (Penn, 2009) but in terms of early cell functions necessary for improved outcome, i.e. attachment and spatial orientation at the injury site, SDF1 preconditioning does not significantly enhance them.

Therefore, moving forward, it is important to understand the adhesion mechanisms involved in human MSCs before blindly testing potential preconditioning factors that have only been shown to work in other cell or species type.

This focus will be on Neuropilin 1 (NRP1), as within the group there is experience with NRP1, but to date there has been no studies identifying it's role for adhesion. NRP1 has been identified as having an important role in the adhesion of endothelial cells and migration of vascular smooth muscles cells (Pellet-Many et al., 2011)

# Chapter 4 – The requirement for NRP1 for attachment, migration and vascular support capacity of human mesenchymal stem cells

## *Introduction*

Bone marrow derived mesenchymal stem cells (MSCs) are multi-potent cells that are currently in clinical trials for cardiac repair. Their expansion capabilities and low immunogenic response are critical attributes for an allogenic therapy. Several studies have shown MSCs to be comparable to peri-vascular cells in both gene and protein marker expression and functionality (Covas et al., 2008b). The previous chapter failed to significantly improve the engraftment or pro-angiogeneic properties of MSCs or MNCs using SDF-1. Therefore, a better understanding of the underlying mechanism of engraftment is required for successful manipulation of the cells.

It is important to understand mechanisms of attachment and migration for MSCs as animal models with MSCs have identified that few cells are retained the peri-infarct region after transplant (Pasha et al., 2008). Approximately 0.5-2% of transplanted cells are identified within this region with quantities diminishing over time (Shake et al., 2002). Research has recently focused on preconditioning cells exploiting the PI3K/AKt survival pathway with factors such as SDF-1 and HGF, however, the previous study was not able to replicate these results using SDF-1 (Gude et al., 2008). Few studies have attempted to investigate adhesion and migratory response after preconditioning with most investigations analysing the effect on survival not function (Pasha et al., 2008). A protein that could be important for adhesion and migration in MSCs is Neuropilin 1 (NRP1).

NRP1 is a known co-receptor of Vascular Endothelial Growth factor receptors (VEGFRs) and Platelet-derived Growth Factor Receptors (PDGFRs) that is vital for attachment and migration in other cell types, including endothelial cells and neurons (Zachary, 2011). NRP1 is a type I transmembrane glycoprotein that regulates vascular development and is a key modulator of α5β1 integrin, a key adhesion molecule for attachment to fibronectin (Zacchigna et al., 2008). Studies in endothelial cells shown NRP1 has a role in angiogenesis (Valdembri et al., 2009),. MSCs express NRP1 and Ball et al, showed that in MSCs cross talk between NRP1 and PDGFRs controls migration, network formation and proliferation (Ball et al., 2010). However, the role of NRP1 for adhesion in MSCs is unknown.

The present study shows that NRP1 is vital for fibronectin mediated adhesion, migration to PDGF-AA and has a role in the pro-vasculogenic function of MSCs

***Hypothesis***

NRP1 expression is required for effective attachment, migration and pro-angiogenic function of bone marrow derived mesenchymal stem cells

***Material & Methods***

All methods and assays are as described in Chapter 2. However due to unavailability of MNC cell samples obtained from the REGENERATE trial this study will only be investigating passage 3 MSCs. This was chosen as 3 passages allows for significant scale of MSCs while keeping functional properties that are lost as the MSCs mature; shown in the previous results chapter

The following methods are applicable to this chapter.

**Transfection with siRNAs**

The role of NRP1 was analysed by knocking down the gene expression, using siRNA. This method had been validated and optimised by Dr. Ian Evans from a collaborating lab at the Department of Cardiovascular Medicine, UCL, under direction of Prof. Ian Zachary.

Transfection of MSCs was performed using Silencer® siRNA targted to NRP-1 (Life Sciences: #4912) or scrambled siRNA and Lipofectamine® RNAiMAX (Invitrogen, UK). MSCs were cultured in 6-well plated (Nunclon, UK) until 50-60% confluent.. Lipofectamine RNAiMAX was diluted with Opti-MEM (Life Sciences, UK) 1:50. siRNA and reagent were mixed and incubated for 20 minutes before adding 500µl of siRNA-Lipofectamine RNAiMAX complexes to each well. Cells were incubated at 37°C, and used experimentally after 24-48hrs.

**Western blot analysis**

Culture media was removed and cells were washed with dPBS then lysed with RIPA buffer (Invitrogen, UK) supplemented with protesase and phosphatase inhibitors (pierce Biotechnology, UK) Cell lysates were separated by electrophoresis on 10% SDS-PAGE gels, and electrotransfered onto PVDF memebranes for one hour at 140V. Membranes were blocked with 5% (w/v) non-fat dried milk in PBS

containing 0.1% tween 20, incubated with NRP-1 (C-19, Santa Cruz, USA) primary antibody (1:200) overnight at 4°C and washed three times in PBS and incubated with horseradish peroxide labelled IgG for 1 hour. Proteins were detected using the ECL PLUS Western blotting detection system and Hyperfilm ECL (Amersham Biosciences). β-actin (1:500, Cat No:04-116,Millipore, UK) was used as a loading control

**Gene expression**

For gene expression studies, after siRNA transfection and stimulation, cells were harvested and pelleted for qPCR as previously described in chapter 2. Neuropilin 1 was accessed using the Quantitect NRP1 primer (Cat no. QT0023009, Quiagen)

**Static adhesion assay**

$1x10^4$ control MSCs (scrambled siRNA transfected) and MSCs target with NRP1 siRNA, were seeded onto 48 plates coated with BSA (2%w/v, Sigma), fibronectin (40µg/ml; R&D systems), vitronectin (2ug/ml;R&D systems), Collagen type I (2µg/ml; BD Biosciences). Plates were incubated at 37°C for either 5 or 20 minutes, then unattached cells were aspirated and wells were washed twice with dPBS. Images in 3 random fields of view in each well were counted at x4 magnification using a Nikon Eclipse microscope. Cells were counted using ImageJ software.

**Migration**

A modified Boydon chamber assay was used to assess cellular migration towards a chemotactic gradient. $1 \times 10^5$ control MSCs or NRP1 Knockdown (KD) MSCs were seeded onto 8µm pore inserts (BD

Biosciences) with either serum free media, SDF-1 (100ng/ml; Biolegend) or PDGF-AA (50ng/ml; R&D Systems) in the lower compartment. Cells were allowed to migrate for 3 hours, after which non-migrating cells were removed from the upper surface of the membrane using a cotton swab. The membrane was fixed with 4% PFA and cells on the underside were stained with 0.25% crystal violet (Sigma) for 30 minutes. 3 random fields of view were imaged under x10 magnification and counted using ImageJ software

**Wound healing assay**

Control MSCs and NRP1 KD MSCs were seeded onto a fibronectin coated 12 well plate and allowed to reach confluence. Subsequently, a small scratch 'wound' was introduced to the cell monolayer using a 200µl pipette tip. The scratch site was imaged and the corresponding scratch location was imaged after 8 hours of incubation. The area of the scratch wound closed was calculated using ImageJ**.**

**Live labelling of MSCs and HUVECs**

To enable visual differentiation between the cell types in culture they were labelled with fluorescing probes with non overlapping emission frequencies.

Adherent HUVECs were labelled by incubating with 10nM CellTracker™ green (Invitrogen, UK; C2925) at 37°C for 30 minutes before washing with culture medium. Adherent MSCs were labelled with DiO (Invitrogen,Uk) for 20 minutes at 37°C before washing with culture medium.

**Tubule formation assay**

Wells of a 96 well Nunc plate were coated with 50µl of growth factor reduced Matrigel® (BD Biosciences) and incubated for 1 hour at 37°C. After knockdown, cells were harvested and resuspended in EGM-2. 1 $x10^4$ control MSCs, NRP1 knockdown MSCs or HUVECS were seeded in EGM-2 medium (Lonza) and incubated for 18 hours. For quantification each well was imaged and cell branch points were counted using ImageJ. Each assay was performed in triplicate.

To assess the interaction of MSCs and endothelial cells within a 3D matrix, $5x10^3$ HUVECs and control MSCs or NRP1 KD MSCs in a 1:1 ratio, were seeded together, and branch points were quantified as described. HUVECs added at 24 hours served as a control.

***In vitro* endothelial network support assay**

To assess the ability of the MSCs to support a pre-existing endothelial network, MSCs were seeded onto a performed tubule network. In brief, 5 $x10^3$ live stained with Celltracker™ (Invitrogen,UK) were seeded onto Matrigel. After 24 hours 5 $x10^3$ HUVECS control MSCs, or NRP1 KD MSCs were seeded into each well. Images were taken of each well after 72 hours. Total branch points were quantified using ImageJ software

## Results

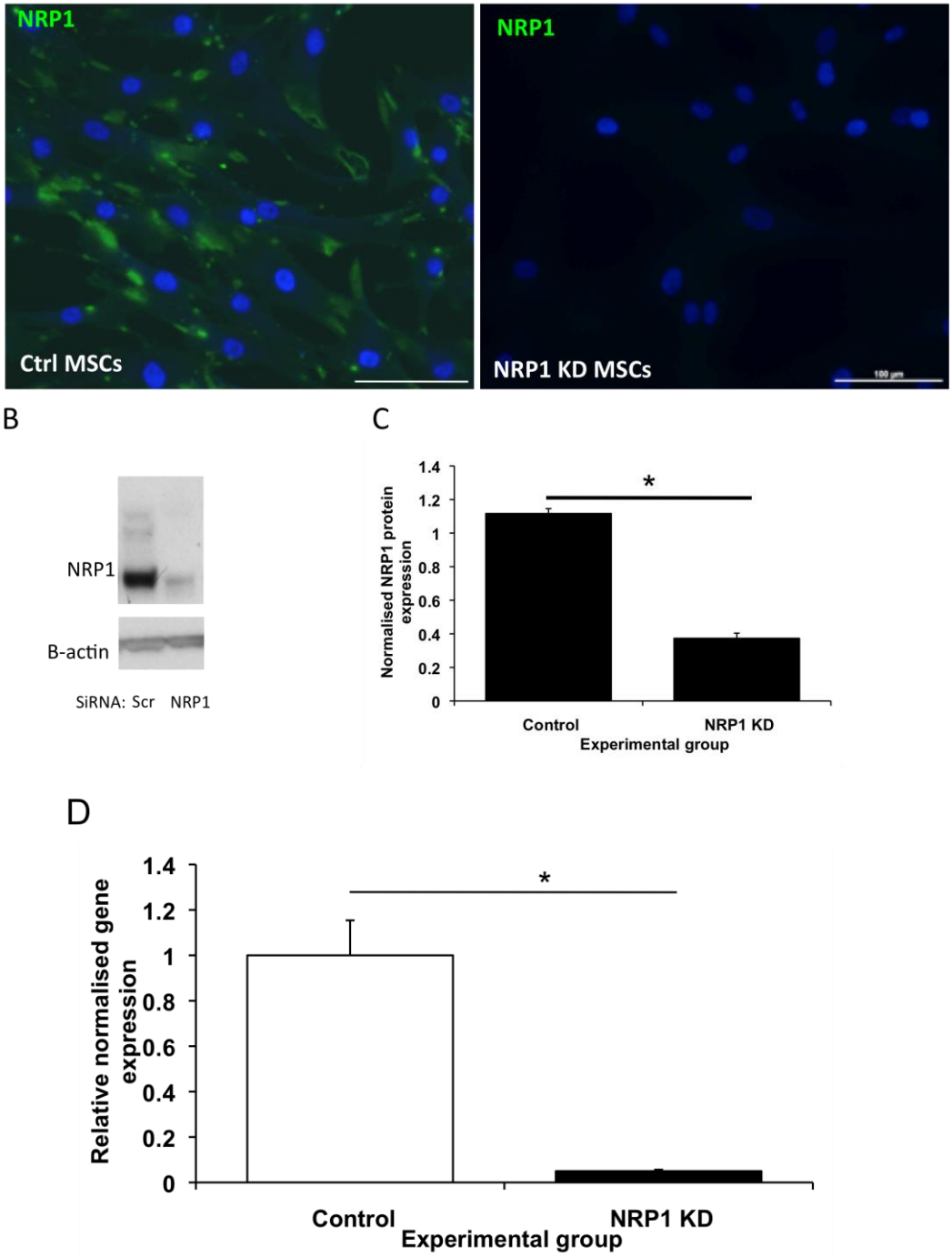



Figure 23 NRP1 knocked down in MSCs by siRNA. MSCs endogenously express NRP1 (A). MSCs were transfected with NRP1 targeted siRNA, or non specific control scrambled siRNA. 24 hours of transfection with siRNA knockdown was confirmed by ICC (A), western blot (B-C) and gene expression of NRP1 according to quantitative qPCR (D). β-actin was used as a loading control for the western blot. Scale bars are equal to 100nm. *=$p<0.05$

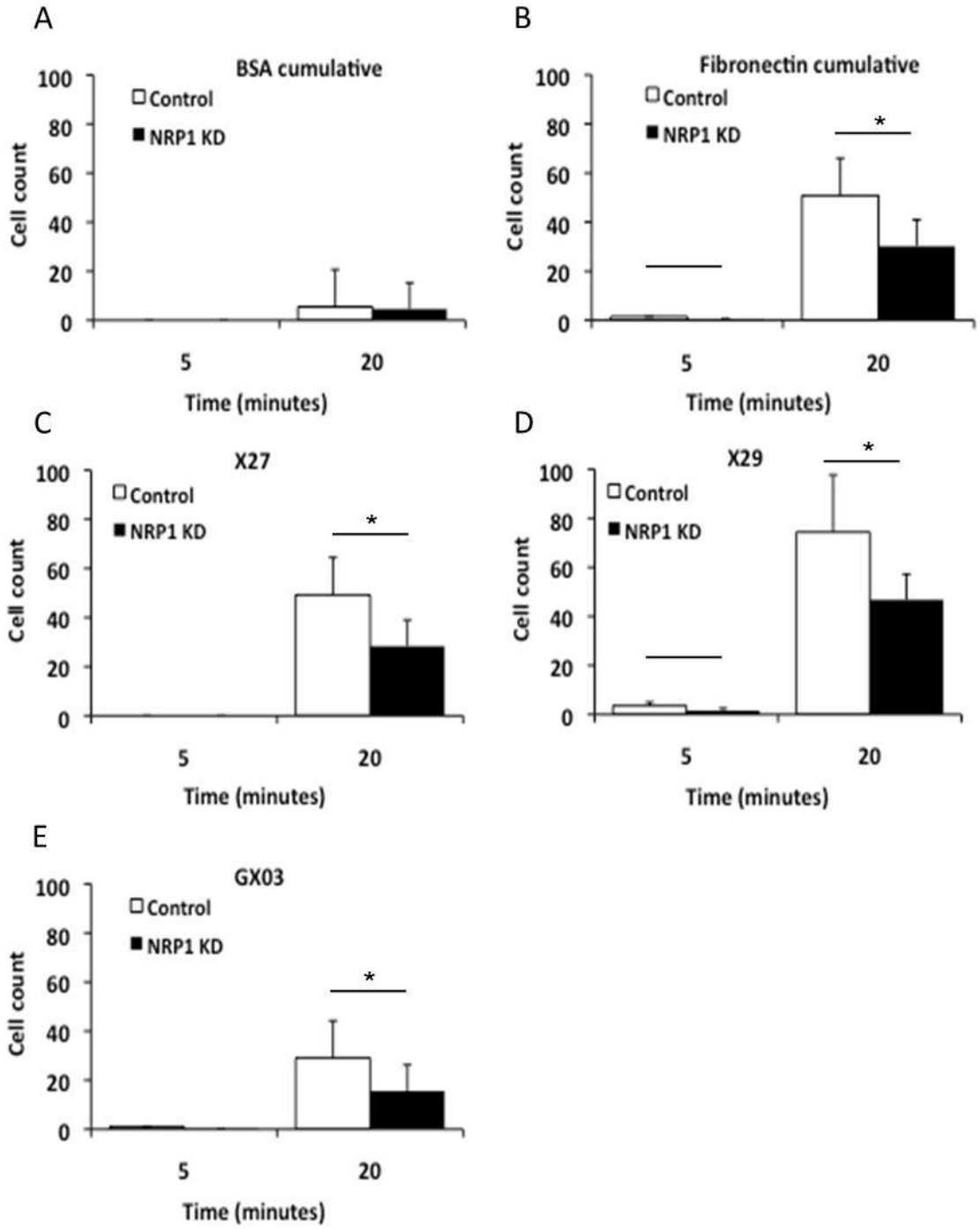



Figure 24 NRP1 plays a role in fibronectin induced attachment. 48 hours after transfection with either scrambled (control) or NRP1 siRNA MSCs were plated onto BSA (A) and fibronectin (B) coated wells for 5 and 20 minutes. Results presented are cumulative data (A-B) from three different donors (C-E). Values are mean number of cells per field view ± SEM (n=3) .*=p<0.05

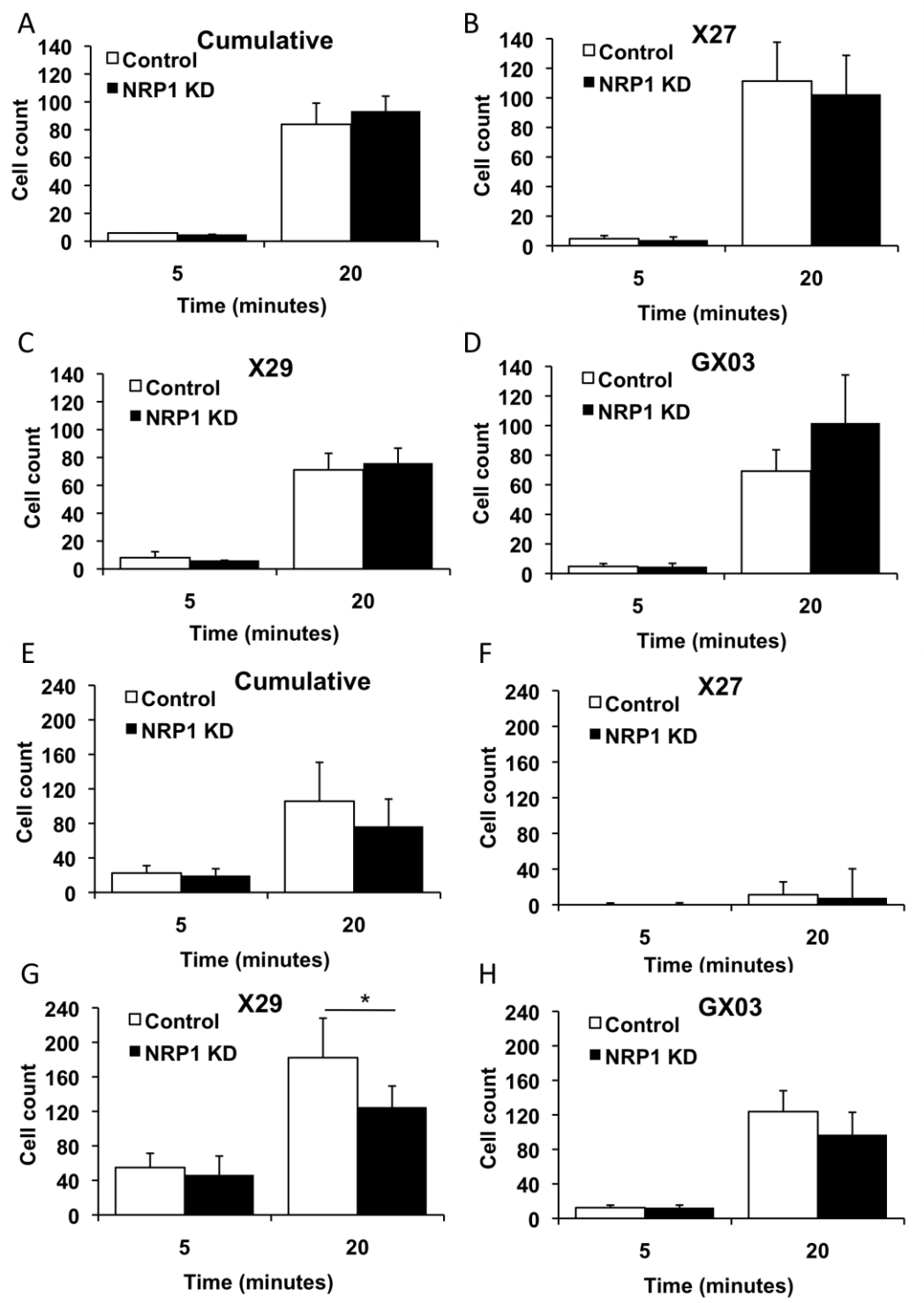



Figure 25 NRP1 not necessary for adhesion to collagen and vitronectin.

48 hours after transfection with either scrambled (control) or NRP1 siRNA MSCs were plated onto type 1 collagen (A-D) and vitronectin -1 (E-H) coated wells for 5 and 20 minutes. Results presented are cumulative data (A,E) from three different donors. Values are mean number of cells per field view ± SEM (n=3) .*=$p<0.05$

**NRP1 is important for adhesion to fibronectin**

MSCs were expanded from isolation in normal growth media that included DMEM, 10% FBS and 1ng/ml bFGF on tissue culture plates. To understand the mechanism that controls adhesion to different ECM proteins, NRP1 was knocked down in MSCs by transfection with a NRP1 targeted siRNA. 24-48 hours after transfection MSCs were used in the experimental assays.

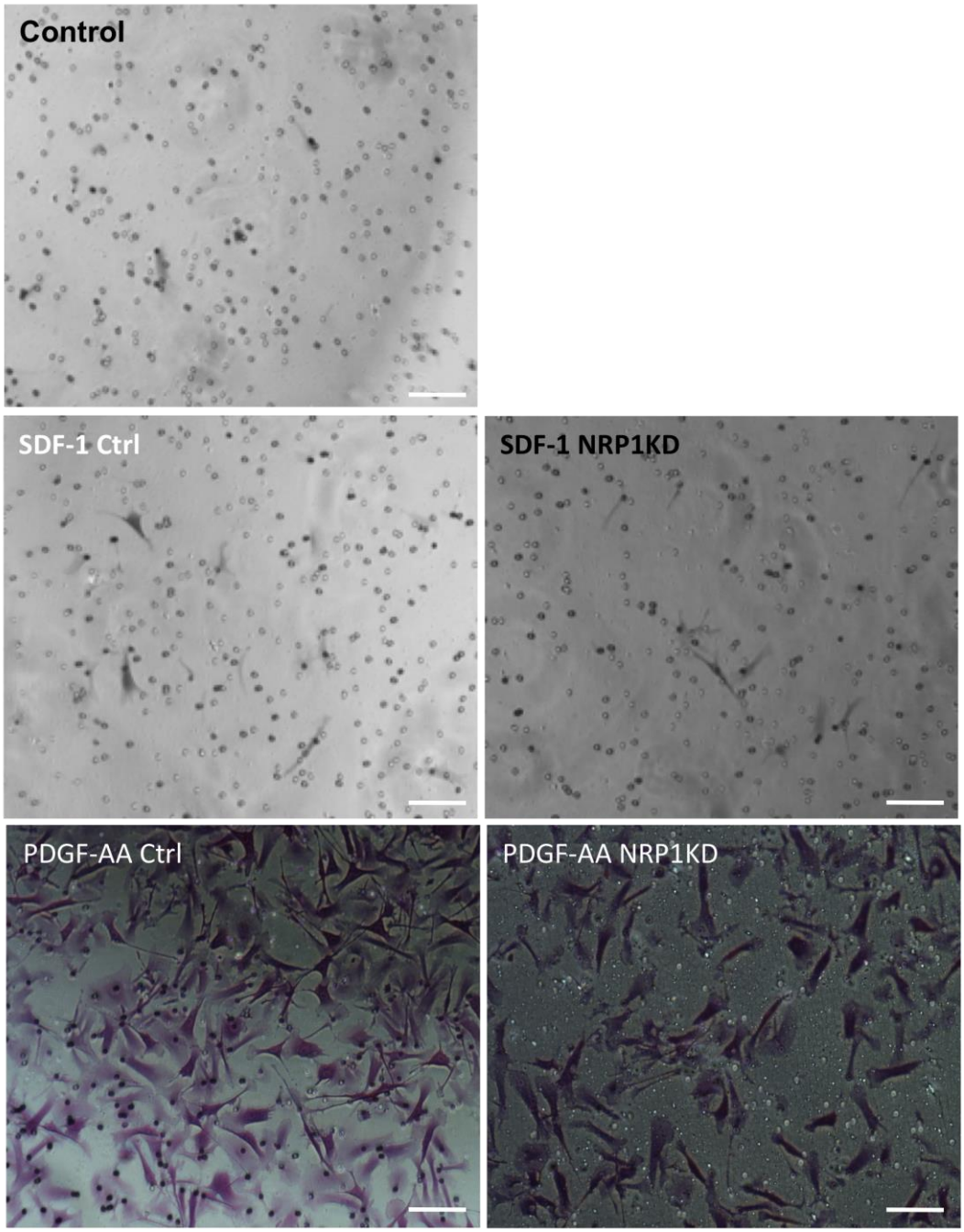
Fibronectin, collagen type 1 and vitronectin 1 are typical ECM proteins, with fibronectin of particular interest as it is highly deposited in the infarct heart. MSCs were seeded onto BSA or fibronectin coated wells and allowed to adhere for 20 minutes. The short term adhesion assays showed that adhesion to fibronectin is significantly reduced by knockdown of NRP1 (Figure 24B) but adhesion to other substrates is not affected (Figure 25 A and E). This is consistent with all MSC isolations except for X29 that has significant decrease in adhesion to vitronectin 1 after NRP1 knockdown (Figure 25G).

**Migration to PDGF-AA, but not SDF-1, is dependent on NRP1**

Having established that NRP1 has an important role in static attachment to fibronectin, chemotaxis towards 100ng/ml SDF1 and 50ng/ml PDGF-AA, was accessed over 3 hours (Figure 26A). Migration index was calculated to express the stimulated migration against random migration in the absence of the chemokines. Migration towards SDF1 was not affected by knocking down NRP1 compared to control MSCs (3.21±0.49 versus 3.09±0.43, $p>0.1$) (Figure 26B).

Exposure to PDGF-AA substantially enhanced migration of control MSCs compared to SDF-1 (17.97±4.67 vs 3.21±0.49). However, knockdown of NRP1 resulted in a significant reduction in migration (17.97±4.67 versus 13.69±3.68) (Figure 26B). This relationship was observed for all independent isolations, although X27 had approximately double the number of migratory cells to PDGF-AA compared to isolations X29 and GX03 (Figure 26C-E). The control migrated MSCs to PDGF-AA appear to display more filopodia than the NRP1 knockdown MSCs (Figure 26A).

A



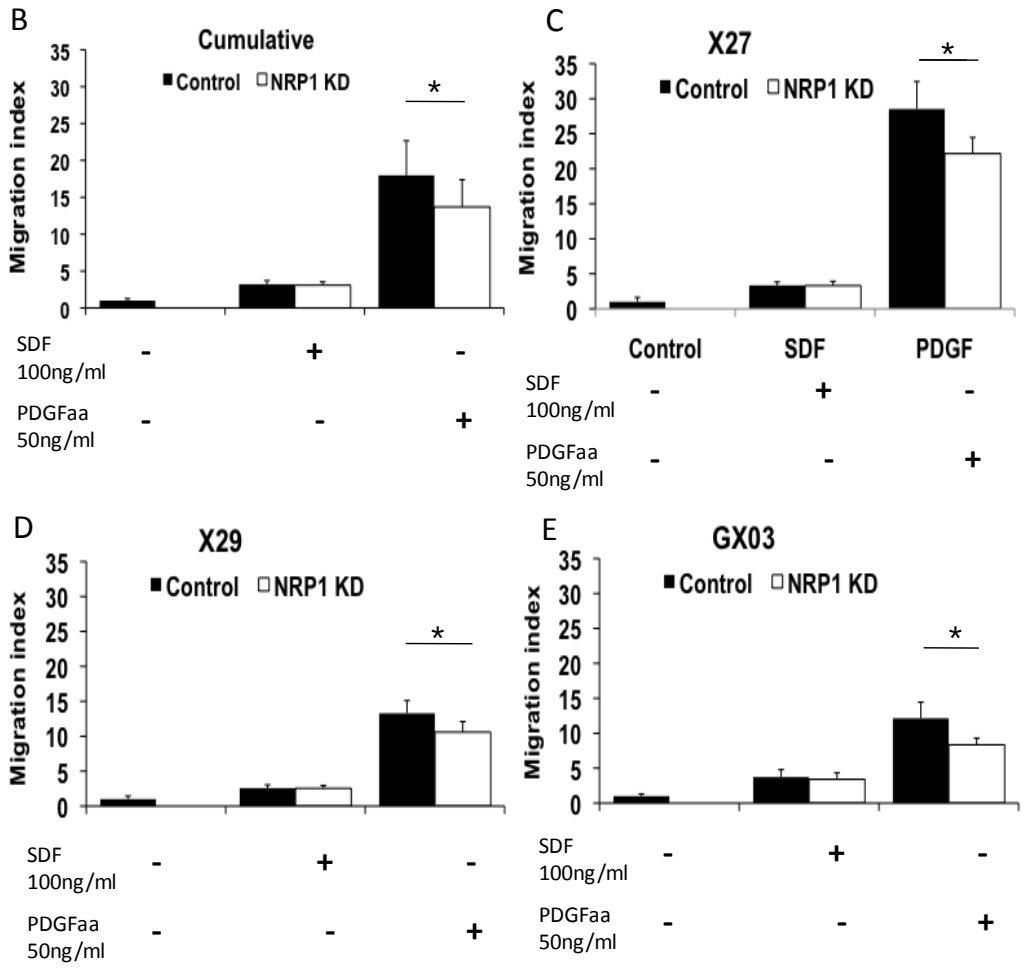



Figure 26 NRP1 has a role in PDGF-AA induced migration. 48 hours after transfection with either scrambled (control) or NRP1 siRNA MSCs were transferred to transwells and allowed to migrate for 3 hours to either serum free media, SDF-1 (100ng/ml), or PDGF-AA (50ng/ml). The transwell membrane were fixed and all non-migratory cells were scrapped off before the migratory cells were stained with crystal violet (Figure A). Scale bars equal 100nm. Results presented are cumulative data (B) from three different donors (C-E). Values are mean number of cells per field view ± SEM (n=3) .*=p<0.05

**NRP1 does not impact wound closure response**

Both control and NRP1 KD MSCs were grown in normal growth media to confluence on fibronectin coated dishes, scratched with a pipette tip and imaged at the start of the experiment and then again after 8 hours (Figure

27A). ‘Scratch area’ was measured for 0hr and 8 hour using Image J to determine closure percentage. The percentage wound closure after 8 hours was not affected by NRP1 knockdown ($p>0.1$) (Figure 27B). There was a large amount of variability in response to the scratch wound between independent donors. Wound closure for donor X27 was slower than for X29, where after 8 hours most wells in both groups exhibited full closure (Figure 27C-E)

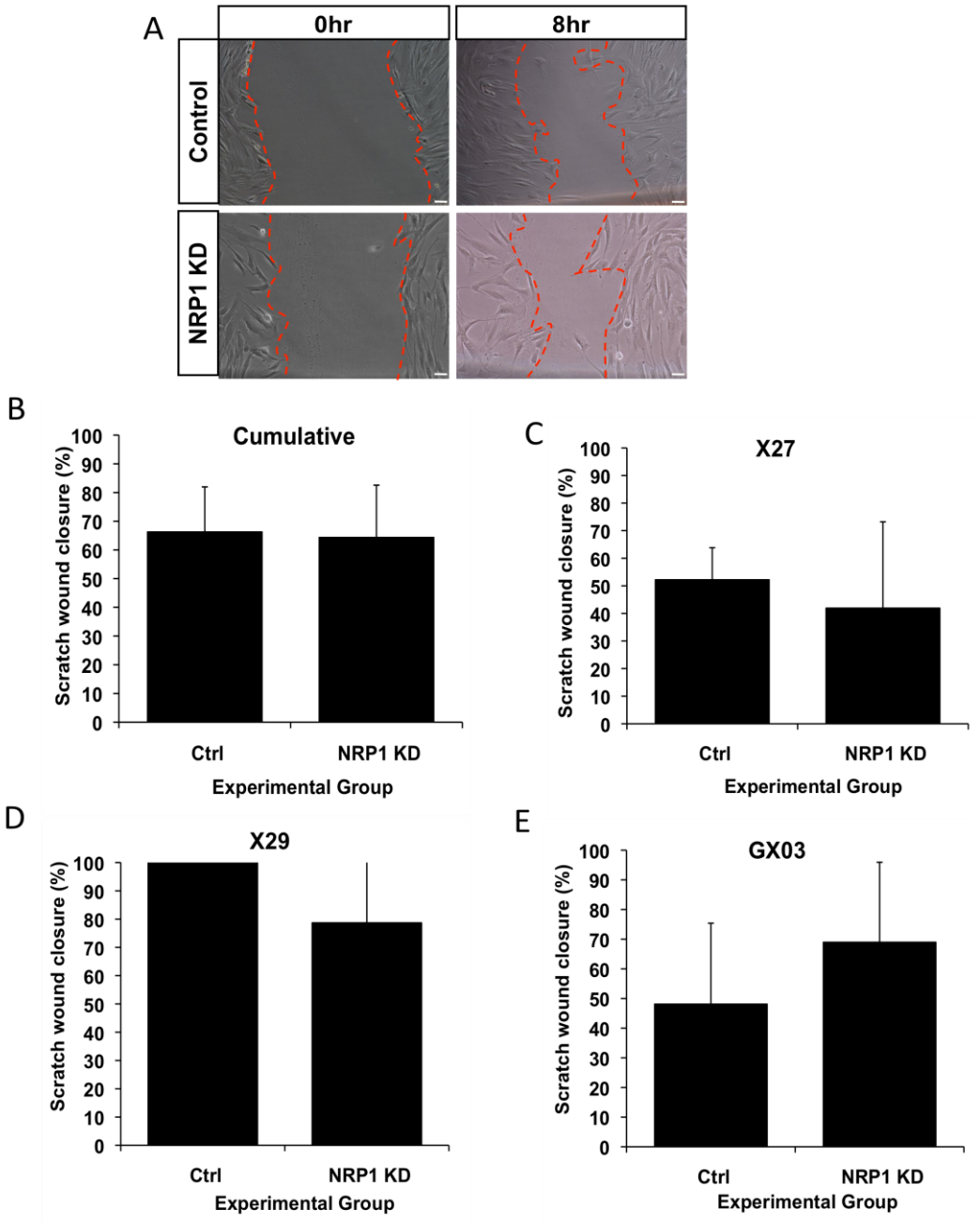



Figure 27 NRP1 does not have an impact on wound healing response. 48 hours after transfection with either scrambled (control) or NRP1 siRNA (control) MSCs were, and grown to confluency. Then a pipette tip was used to create a scratch, the closure of the scratch wound was observed over 8 hours (A). Dotted red line indicates scratch wound edge. Values are mean scratch wound closure ± SEM (n=3) (B-E).

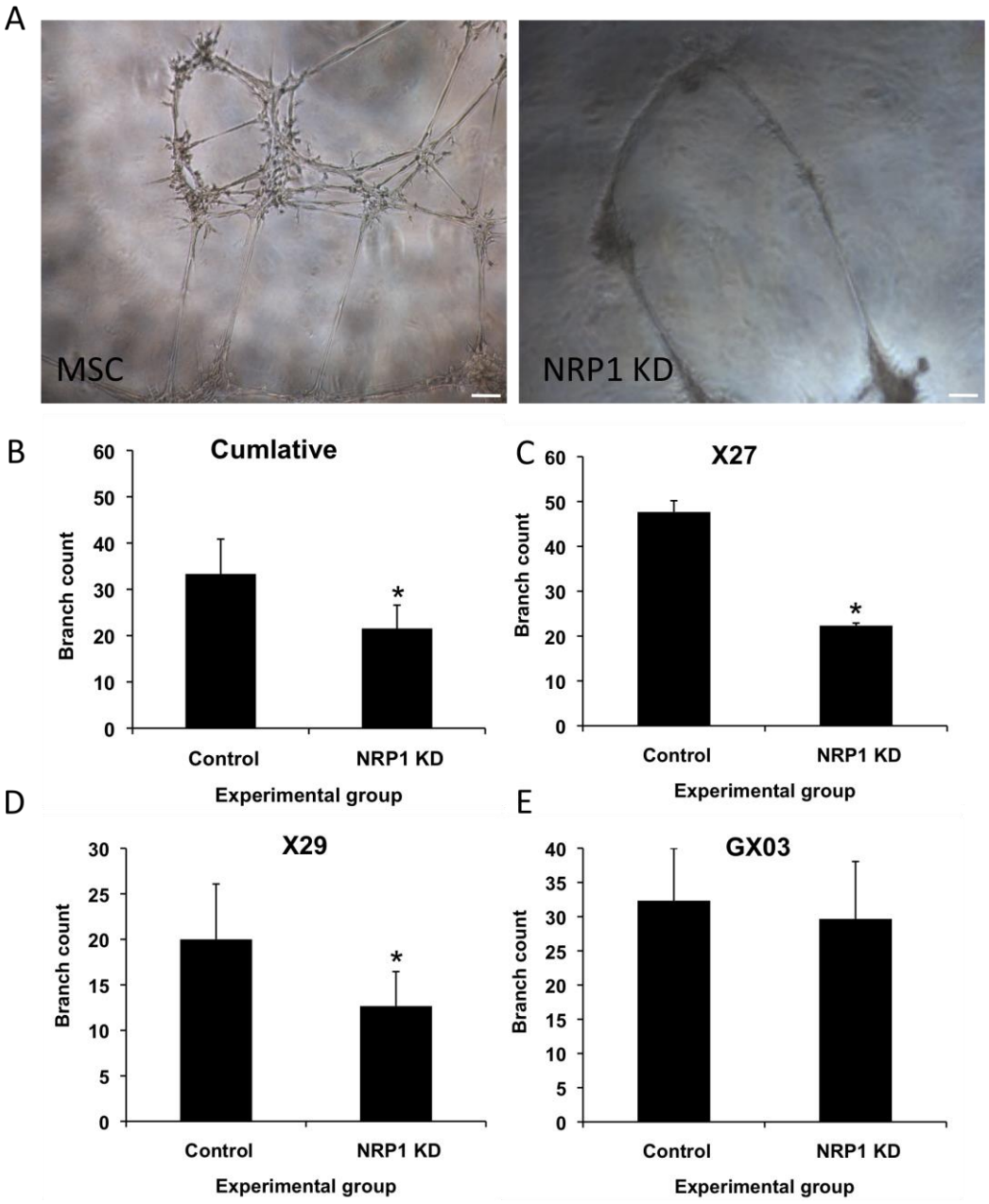


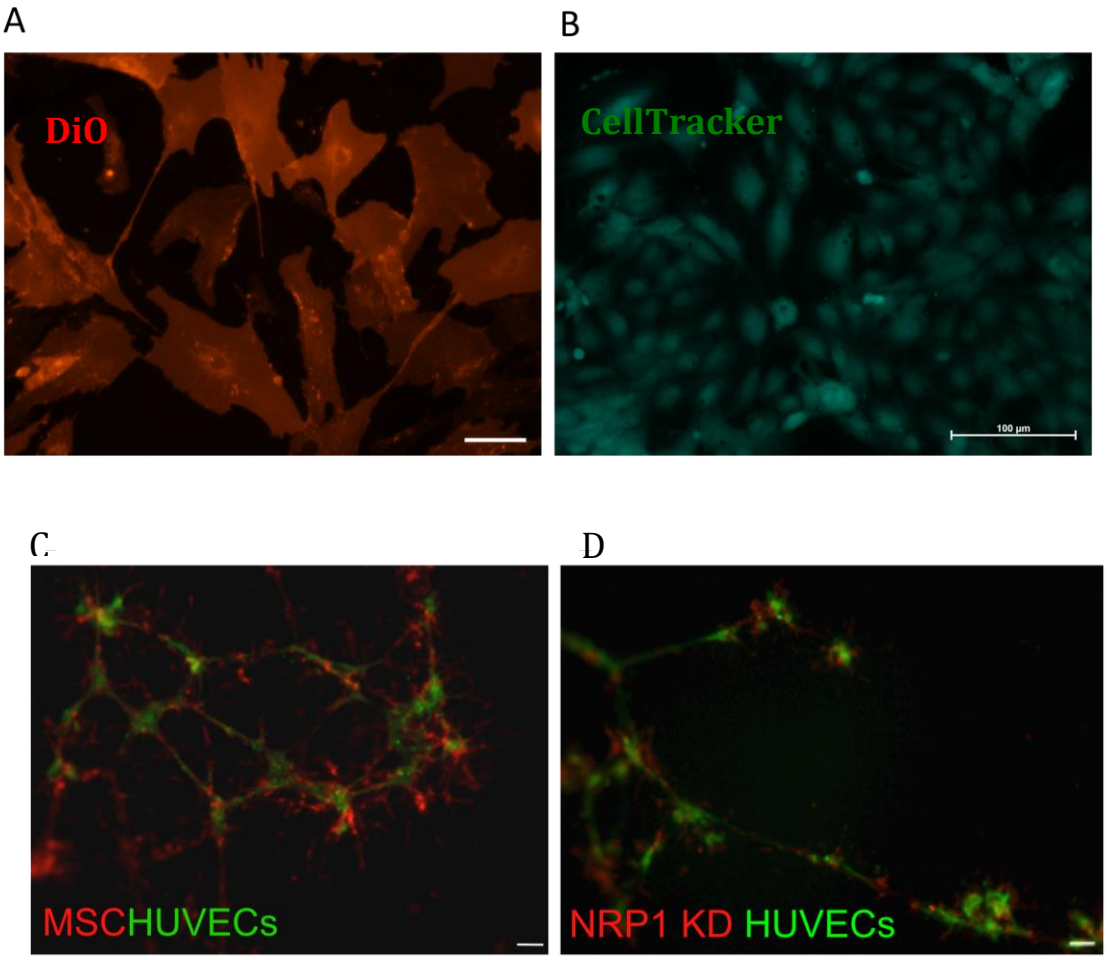

Figure 28 The effect on NRP1 knockdown on network formation was examined using an *in vitro* Matrigel assay. MSCs were seeded onto the gel and incubated for 18 hours (A).. Results presented are the total the branch points formed through the entire well (B-E). *=$p<0.05$. Scale bar represents 100nm

**MSCs form a tubule network in matrigel**

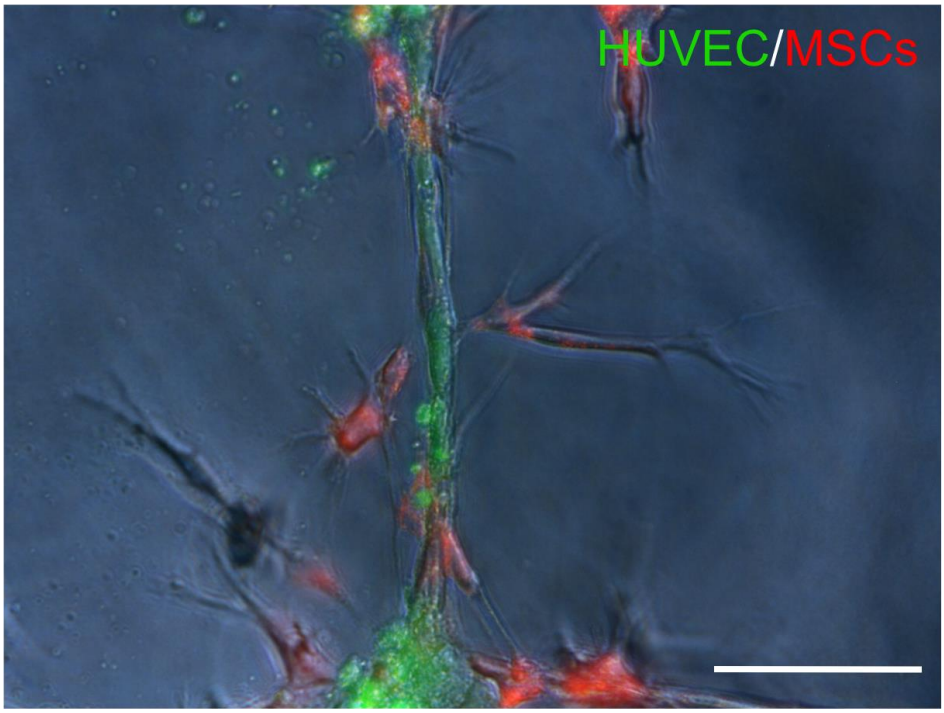
To test the hypothesis that MSCs have pro-angiogenic properties that are measurable in simple *in vitro* assays, several Matrigel-based assays were set up. MSCs were seeded either alone or in a co-culture with HUVECs to determine the requirement of NRP1 in cell to cell over 18 hours. 1 $x10^4$ MSCs in EGM2 were seeded onto a thick matrigel gel coating (Figure 28A). EGM-2 contains a number of growth factors and supplements including When NRP1 was knocked down, MSCs formed fewer branchpoints than control MSCs (Figure 28B), Moreover, NRP1 KD MSCs agglomerated at branch points (Figure 28A).

**MSCs stabilise an endothelial network in a pericyte-like function**

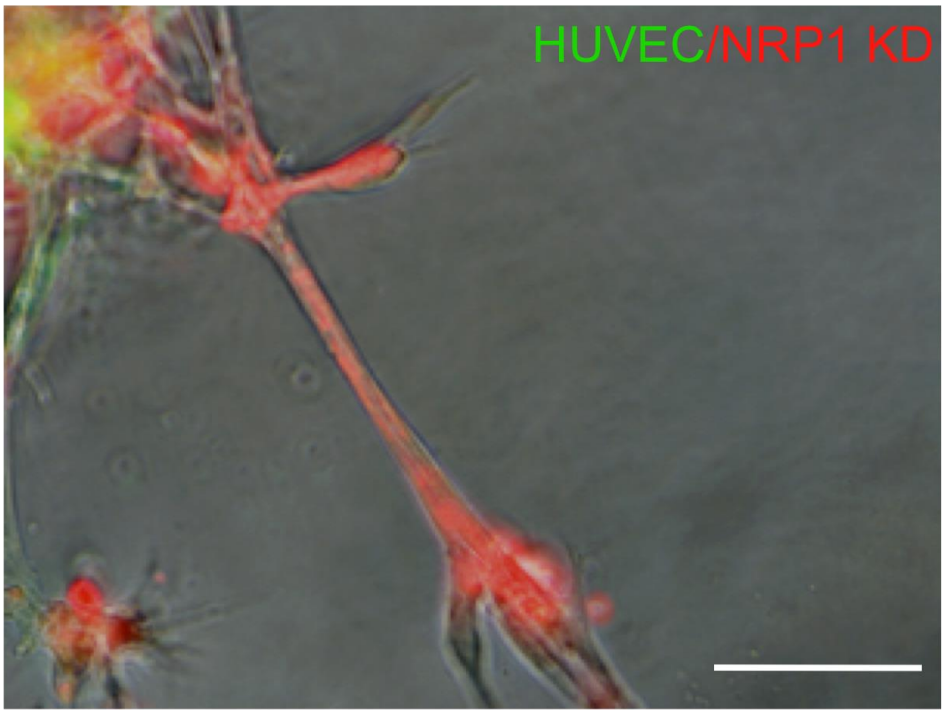
To understand how MSCs would interact with an endothelial network upon delivery to a physiological site, MSCs were seeded onto a pre-existing HUVEC network. MSCs were stained with a fluorescent marker, DiO (Figure 29A) and HUVECs with Celltracker (Figure 29B), to identify each cell type. HUVECs formed a lattice like network after 24 hours. When MSCs were added at this time point the network was stabilised for at least 72 hours (Figure 29C). In contrast, the network was not stabilised when HUVECs or NRP1 knockdown MSCs were added (Figure 29H) and resulted in significantly decreased branch points. Live cell staining of the MSCs and HUVECs revealed physical interaction between the 2 cell types (Figure 29D-G) but this was diminished when NRP1 KD MSCs were used, with MSCs mostly seen agglomerated at branch points (Figure 29G). After 72 hours the MSCs appeared to support the HUVEC network bridging between tubule forming endothelial cells (Figure 29F).


A
DiO
B
CellTracker
100 µm
C
MSCHUVECs
D
NRP1 KD HUVECs
E
MSCs
NRP1 KD
HUVECs
24hrs
72hrs

F




G



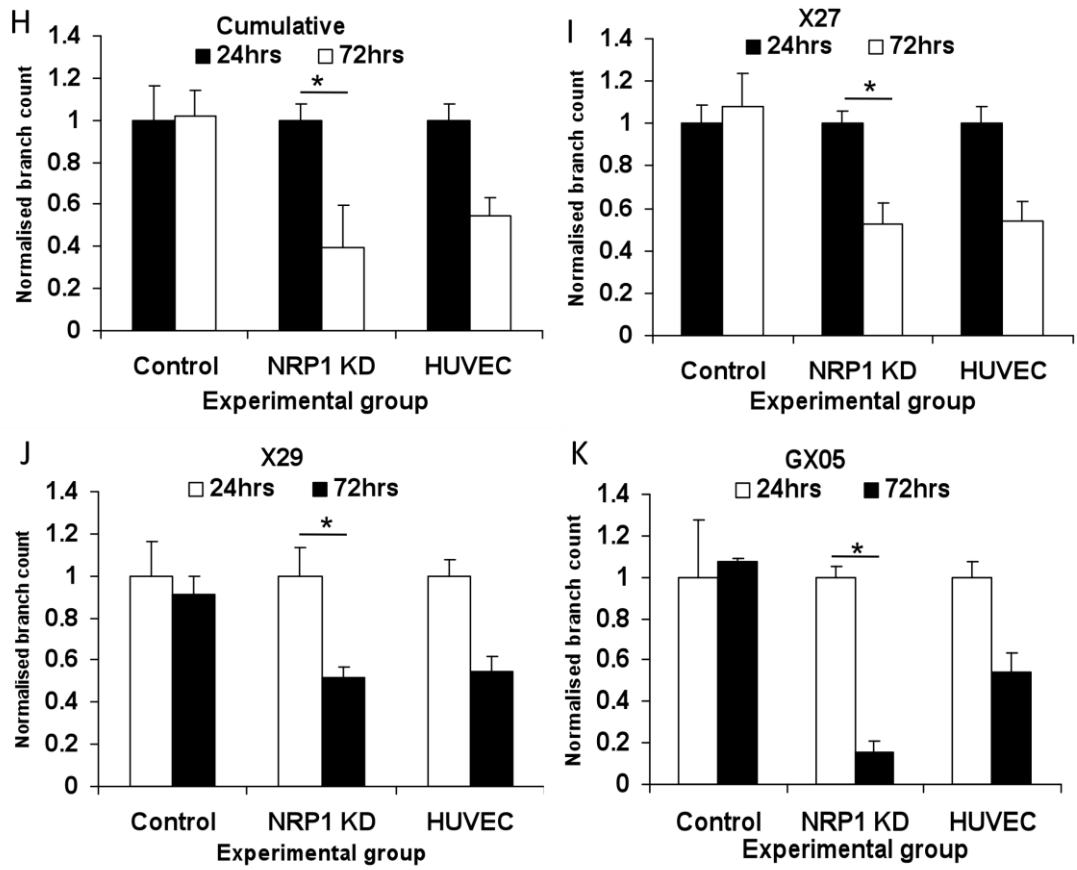



Figure 29 – NRP1 expression in MSCs is necessary to support an endothelial network. 48 hours after transfection with either scrambled or NRP1 KD siRNA MSCs were seeded onto a HUVEC network on a matrigel layer. The HUVEC network was created by seeding HUVECs in matrigel for 24 hours. Live cell staining of MSCs (A) and HUVECs (B) using fluorescent probes DiO (red) and Celltracker (green) respectively. Images were taken at 24 and 72 hours (C-E), Fluorescent images were taken at x4 (figure C-D) and x20 magnification (F-G). Scale bar represents 100nm Results presented are normalised branch points of the entire well to their respective quanity at 24 hours + SEM (H-K). *=$p<0.05$.

**Endothelial cells form tubules on a MSCs monolayer**

HUVECs were seeded onto a MSC monolayer and probed for PECAM-1, an endothelial marker. They formed tubule like structures on the control MSCs (Figure 30C) but not on top of the NRP1 KD MSCs where they formed random structures and agglomerates (Figure 30D). Phase contrast images showed no structural appearances (Figure 30A-B)

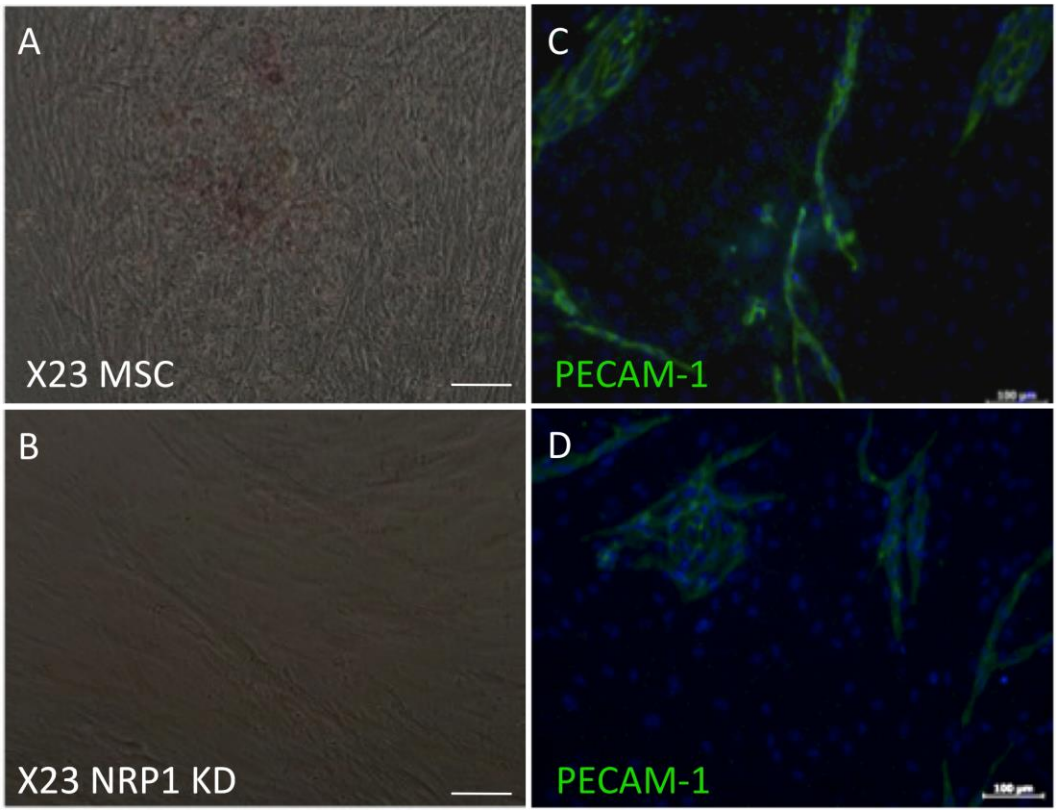



Figure 30 HUVECS form networks on a monolayer.

MSCs, knockdown and control, were grown to confluency in 24 well plates. HUVECs were added onto the monolayer and incubated for 72 hour (A-B). Cells were then fixed and probed for PECAM-1 by immunocytochemistry (C-D). Images were taken at x4 magnification. Bars represent 100um

## Discussion

In the previous chapter, it was shown that SDF-1 was unable to improve the engraftment responses of bone marrow derived mononuclear and MSCs. To improve the functional characteristics of cell therapy candidates the mechanism underpinning attachment and migration needed to be understood. One key limitation in this chapter was the lack of availability of mononuclear cells from the clinical trial to perform additional analysis. However, it may have been difficult to work with mononuclear cells as maintaining them in suspension culture for the duration of the knockdown protocol would have substantially reduce cell viability and impacted on the validity of the data.

Identifying the proteins that are crucial for attachment, migration and general tissue support at sites of ischemic injury is fundamental to the improvement of cell therapy strategies for myocardial infarction. Integrins are known surface receptors that interact with ECM ligands but there are other surface receptors with less characterised roles. The function of NRP1 has been much investigated in other cell types such as endothelial and smooth muscle cells, but only one to date in MSCs (Ball et al., 2010; Pellet-Many et al., 2011; Valdembri et al., 2009). In this study the functional role of NRP1 during attachment, migration and vascular support responses by MSCs was investigated.

This study showed that NRP1 is important for attachment of MSCs specifically to fibronectin, but not to other tested ECM components, and is in part required for migration towards PDGF-AA, but not SDF1. Also, the

ability for MSCs to support an endothelial network *in vitro* is diminished in the absence of NRP1

MSCs endogenously express NRP1, and so to assess its effect on functional characterstics it was knocked down using siRNA. Immunofluroscence imaging revealed that although NRP1 is described as a transmembrane protein the majority is expressed intracellularly. This is consistent with, a recent study by Ball et al study who also show that MSCs express NRP1 and similar to immunofluroscence images presented in this chapter, NRP1 was mainly located intracellularly (Ball et al., 2010).

After myocardial infarction, fibronectin is deposited within the peri-infarct zone of the myocardium (Ip et al., 2007). MSCs rapidly adhere to fibronectin via integrins (primarily $\alpha 5\beta 1$). However, given the overall hostile environment in the post-infarct heart, cell attachment, and survival are low (Pasha et al., 2008). By improving these 'engraftment' characteristics, has the potential to improve MSC survival and therapeutic efficacy.

Other ECM components within the myocardium also include type 1 collagen and vitronectin, as these are also ECM components within the myocardium. The different ECM proteins have have key interactions with different integrins receptors: fibronectin is associated with α5β1 and αVβ3; victronectin 1 with α3β1; and type 1 collagen with α1β1/ α2β1(Covas et al., 2008b).

After successfully knocking down NRP1 using siRNA, the ability of MSCs to attach to fibronectin significantly decreased, but attachment to type I collagen and vitronectin was not affected. This is not surprising, as in

endothelial cells NRP1 was previously reported to interact with active α5β1 at sites of adhesion (Valdembri et al., 2009). Other studies on MSC adhesion reported that α5β1 significantly increased the tyrosine phosphorylation of PDGFR following ligation with fibronectin (Veevers-Lowe et al., 2010). In spite of the evidence to demonstrate that NRP1, α5β1, and PDGFRs are implicated in attachment to fibronectin, however there are no studies have confirmed the mechanism of their combined interaction. Knockdown of NRP1 did not completely impair adhesion of MSCs to fibronectin. While integrin α5β1 is a key integrin for fibronectin, αVβ3 also plays an important role and there is no current evidence to show interaction with αVβ3 and NRP1 (Ip et al., 2007). Therefore, functional rescue by αVβ3 may explain why attachment was not completely impaired

PDGF-AA is released post myocardial infarction, as is SDF-1 (Wallace et al., 1998, {Ip, 2007 #90)}. Therefore, the importance of NRP1 for migration in response to NRP1 and PDGF-AA was investigated using a transwell chemotaxis assay. The first important observation was that MSC migration towards PDGF-AA was much greater than towards SDF-1. Secondly, NRP1 knock-down resulted in significantly decreased migration towards PDGF-AA but not SDF-1. This corroborates with a previous study that assayed the chemotactic effects of 16 different growth factors and chemokines on human bone marrow-derived mesenchymal cells, where PDGF was the most potent and chemokines such as SDF-1 invoked limited responses (Ponte et al., 2007).

The link between NRP1 and PDGF-AA has been documented previously. Ball et al also showed that knockdown of NRP1 decreased migration of MSCs to PDGFR-AA (Ball et al., 2010). NRP1 is also important for migration of other cell types including endothelial cells and vascular cells towards PDGF-AA (Pellet-Many et al., 2011; Valdembri et al., 2009). The inhibition of PDGF-AA responsiveness due to NRP1 knockdown is mediated, at least in part, by $p130^{cas}$ (Pellet-Many et al., 2011). The cross talk between NRP1 and PDGFR that controlled not only migration, but also signalling and proliferation confirming that NRP1 has multi functional roles (Ball et al., 2007). Like adhesion, for migration to PDGF there is interplay between α5β1 integrin activated PDGFRs.

Despite a significant decrease in cells attached to fibronectin, no effect was observed in a wound response assay on fibronectin after NR1 knockdown. This was susprising, as previous studies reported that pre-treatment of endothelial cells with recombinant NRP1 significantly improved wound repopulation (Uniewicz et al., 2012). Moreover, NRP1 mediated endocytosis and recycling of α5β1 integrin to the cell's leading edge during endothelial migration on fibronectin has been reported (Valdembri et al., 2009). However, there are multiple limitations to this assay that may explain our inability to delineate a difference. Firstly, the 'rate' of wound repopulation was not determined in real time, rather single photomicrographs were taken at the start of the assay and at 8hrs. Secondly, due to the manual production of 'scratch' wounds, reproducibility is poor, as reflected by large standard deviations. Thirdly, the fibronectin layer may be partly or completely removed when the cell

monolayer is scratched. Finally, there may be no difference when NRP1 Is knocked down simply because migration is initiated via functionally competent αVβ3 and α8β1, which are not been reported to be dependant on NRP1 (Charo et al., 1990; Marshall et al., 1995)

Tubule formation assays utilising ECM substrates rich in basement membrane proteins, such as Matrigel, have been extensively utilised. All assays in this chapter were conducted using EGM-2 a proprietary growth media supplemented with pro-angiogenic factors including VEGF, EGF, and IGF at undisclosed concentrations. MSCs lacking the supplement, no vessel-like networks were observed, confirming the inter-dependence on these factors and the Matrigel. The formation of tubules seen when plating WT MSCs confirmed the ability of MSCs to respond to vascular stimulus and represents a balance between cell-cell interactions and cell extension and migration across the Matrigel. When NRP1 fewer branches were formed, consistent with findings from Ball et al (Ball et al., 2010) In addition NRP1 knockdown, did not eliminate tubule-like networks completely, but cell clustering around branch points was noted.

Vascular support is hypothesised to be a potential mechanism of action of MSCs on delivery to ischemic tissues. This is particularly desired in an indication like myocardial infarction as not only are cardiomyocytes lost, but also the vasculature that is needed to support the cells in the peri-infarct region. Therefore, understanding how MSCs interact with a pre-existing endothelial network is crucial. Using a modified method from a previous study with dental pulp cells (Dissanayaka et al., 2012), MSCs were seeded onto a pre-exisiting endothelial network and incubated for a

furthur 48 hours. Direct co-culture of MSCs increased the longevity of pre-exiting. However, when NRP1 was knocked down, the network substantially diminished. Adding HUVEC alone to pre-existing vessel like structures did not support vessel stabilisation did not occur. The MSCs were observed to bridge between the endothelial cells and make physical contact in a manner akin to pericyte cells, a phenomenon that was lacking in the absence of NRP1. This provides evidence of a mechanism of pro-vascular support by MSCs, governed not solely by paracrine factors, and dependant on direct contact and interaction with endothelial cells.

When HUVECs were co-cultured on a monolayer of MSCs distinct PECAM-1 positive vessels were formed. However, HUVECs cultured on the NRP1 knockdown MSCs tended to form more sporadic and less tubules-like structures, reinforcing the importance of NRP1 for MSC cell to cell contact with endothelial cells for tubule formation.

As identified in the previous chapter, in spite of the inter-donor variability (notably in the wound repopulation assay), the experimental observations were, largely, highly conserved between different donors. It was notable that isolation X27 had the highest migratory response to PDGF-AA, diminished response in the wound assay, and high branch count in the Matrigel assay, Many other studies have only identified donor differences for proliferation and differentiation potential (Lo Surdo and Bauer, 2012).

***Conclusion***

In conclusion, data in this chapter has revealed that NRP1 is important for adhesion to fibronectin, migration to PDGF-AA, and vascular support

responses. This data provides the foundation for exploration of novel methods by which to stimulate and enhance engraftment of MSCs at physiologic sites. Continuation of this research, in the next chapter, focused on determining whether NRP1 can be up-regulated by preconditioning the cells, using a strategy that is accessible to cell therapy bioprocessing.

# Chapter 5 – Effect of hypoxia and bFGF on Neuropilin 1 expression, cell retention responses and pro-vasculogenic properties of MSCs

## Introduction

Preconditioning strategies have been used in multiple animal AMI models to successfully improve cardiac output, including pharmacological stimulation, or environmental stimulus such as hypoxia (Pasha et al., 2008) (Wisel et al., 2009). The purpose of preconditioning is typically to activate endogenous pathways that have multi-factorial effects including increased survival, enhanced cytokine release, or improve homing to the physiologic site of interest.

Hypoxic preconditioning, has been found to increase MSC survival in ischemic environments after transplantation. In these low oxygen conditions VEGF and HGF, important growth factors for angiogenesis and cytoprotection, become upregulated in MSCs (Chang et al., 2012). As data in chapter 4 indicates NRP1 is important for cell adhesion, we wanted to determine whether it is regulated by oxygen tension. It is already known that the effect of hypoxia on NRP1 expression is cell dependent, so whilst NRP1 In endothelial cells and embryonic cells is upregulated by hypoxia, it is downregulated in neuroblastoma cell lines (Brusselmans et al., 2005; Misra et al., 2012; Ottino et al., 2004)

NRP1 expression is regulated by bFGF in vascular smooth muscles cells that undergo increased chemoxtaxis to VEGF-A in a NRP1 dependent

fashion (Liu et al., 2005). Furthermore, bFGF, along with IGF-1 and BMP-2 illict a pro-survival effect of MSCs (Hahn et al., 2008). Treatment with this growth factor combination enhanced the MSC cardiac differentiation efficiency and cytoprotection of co-cultured adult cardiomyocytes (Hahn et al., 2008). Importantly, cytoprotection was dependent on gap-junction communication with cardiomyocytes, demonstrating the need for direct interaction between the MSCs and cardiomyocytes, rather than by a paracrine mechanism. Similarly, Khan et al preconditioned MSCs isolated from diabetic mice with a combination of IGF-1 and bFGF and showed enhanced cell survival, proliferation, motility and angiogenic potential compared to untreated cells or cells treated with single growth factor (Khan et al., 2011). Therefore, whether bFGF can enhance NRP1 and improve functional characteristics attributable to NRP1 is an interesting research question

The previous chapter showed that NRP1 is an important protein in MSCs for adhesion to fibronectin, migration to PDGF-AA and vascular support responses. In the present study, strategies to upregulate NRP1 expression using hypoxia and bFGF were investigated, and the effect on the MSC adhesion, migration and cytokine release was quantified

**Hypothesis**

Enhancing the expression of NRP1, by preconditioning the MSCs, is positively correlated with improved adhesion, migration of MSCs and vascular support responses.

***Materials & Methods***

Culture of MSCs and functional assays were as described in Method and materials. Except for the following additions and adaptations

**bFGF stimulation**

MSC experimental groups were set up as follows

1. Control MSCs (scrambled siRNA), passage 3, unstimulated
2. Control MSCs (scrambled siRNA), passage 3, stimulated for 12 hours with 10ng/ml bFGF
3. siRNA NRP1 knockdown MSCs, passage 3, unstimulated
4. siRNA NRP1 knockdown MSCs, passage 3, for 12 hours with 10ng/ml bFGF

**Gene expression**

For gene expression studies, after siRNA transfection and stimulation, cells were harvested and pelleted for qPCR as previously described in chapter 2. Neuropilin 1 was accessed using the Quantitect NRP1 (Cat no. QT0023009, Quiagen)

For the differentiation assays, actinin-associated LIM protein (ALP) and peroxisome proliferator-activated receptor gamma (PPARg) Quantitect primers (Cat no. QT01905099 & QT0005468, Quiagen) were used as according to manufacturer's instructions in the two step qPCR protocol

**Migration**

A modified Boydon chamber assay was used to assess cellular migration towards a chemotactic gradient. 1 x $10^5$ MSCs were seeded onto 8μm pore inserts (BD Biosciences) with either serum free media (control) or PDGF-AA (100ng/ml; R&D Systems) in the lower compartment. Cells were allowed to migrate for 3 hours, after which non-migrating cells were removed from the upper surface of the membrane using a cotton swab. The membrane was fixed with 4% PFA and cells on the underside were stained with 0.25% crystal violet (Sigma) for 30 minutes. 3 random fields of view were imaged under x10 magnification and counted using ImageJ software

**Cytokine release**

MSCs were seeded at 1 x $10^4$ in 6 well plates in duplicate for each experimental group. After the siRNA transfection protocol, as described in chapter 4, groups 2 and 4 were treated with 10ng/ml bFGF. Then media for all cells was replaced with normal MSC growth media with only 1% FBS. After 24 hours conditioned media was obtained and measured using commercially available Quantikine® enzyme linked immunosorbent (ELISA) assays for VEGF, IL-6, HGF, and PDGF-AA (DVE00, D6050, DHG00, DAA00B; R&D Systems). Each measurement was standardised to reference concentrations of protein as per manufacturer's instructions.

### Proliferation

To investigate the proliferation effect of bFGF over 24 hours, $1x10^4$ MSCs were seeded in 96 well plates. The cell number was measured using Cell Counting Kit-8 (Dojindo, Japan). MSCs were mixed with 10µl of CCK-8 solution per well and incubated for 1 hour at 37°C. The plate was then read at an absorbance of 450nm using a microplate reader. Each experimental group was repeated for three different isolations in triplicate wells.

## *Results*

### NRP1 expression differentially regulated by hypoxia and bFGF

In the previous chapter, we reported that NRP1 has a role in optimised cell attachment and migration. Here we assessed the effect of hypoxia on NRP1. A few studies have shown NRP1 to be over-expressed in different cell types by a number of strategies, including hypoxia preconditioning and bFGF stimulation (Liu et al., 2005). Using mRNA quantification, we found that low oxygen significantly reduced NRP1 expression in MSCs after 1 hour ($p=0.006$) and furthermore at 24 hours in 2% oxygen (Figure32A). The next strategy was to stimulate the MSCs with 10ng/ml bFGF for 12 hours. mRNA expression of NRP1 was significantly over-expressed in

MSCs (Figure 32A). This was consistent in all isolations used (Figure 32B).

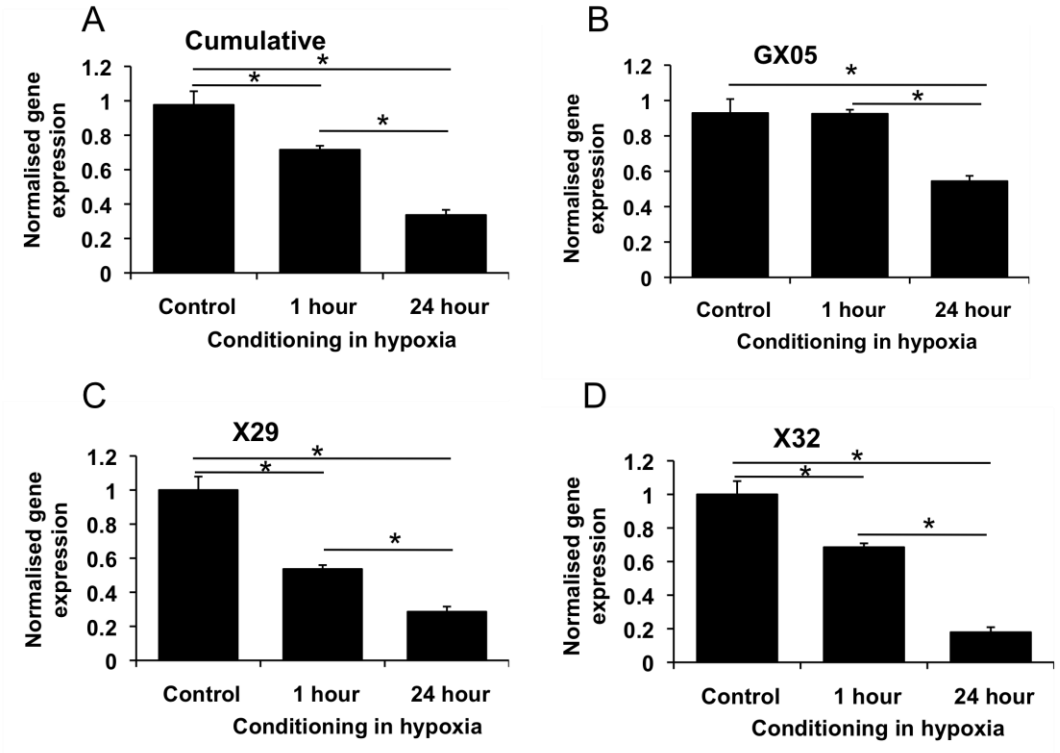



Figure 31 - NRP1 is down regulated by hypoxia

MSCs were put in a hypoxic chamber purged with 2% oxygen and incubated for 1 or 24 hours, or in control conditions of 20% oxygen. Cells were harvested and the mRNA isolated. Results presented are cumulative data (A) from three independent donors (B-D). Values =Gene expression for NRP1 analysed by qPCR and normalised to GAPDH & control group ± SEM (n=3) *=$p<0.05$

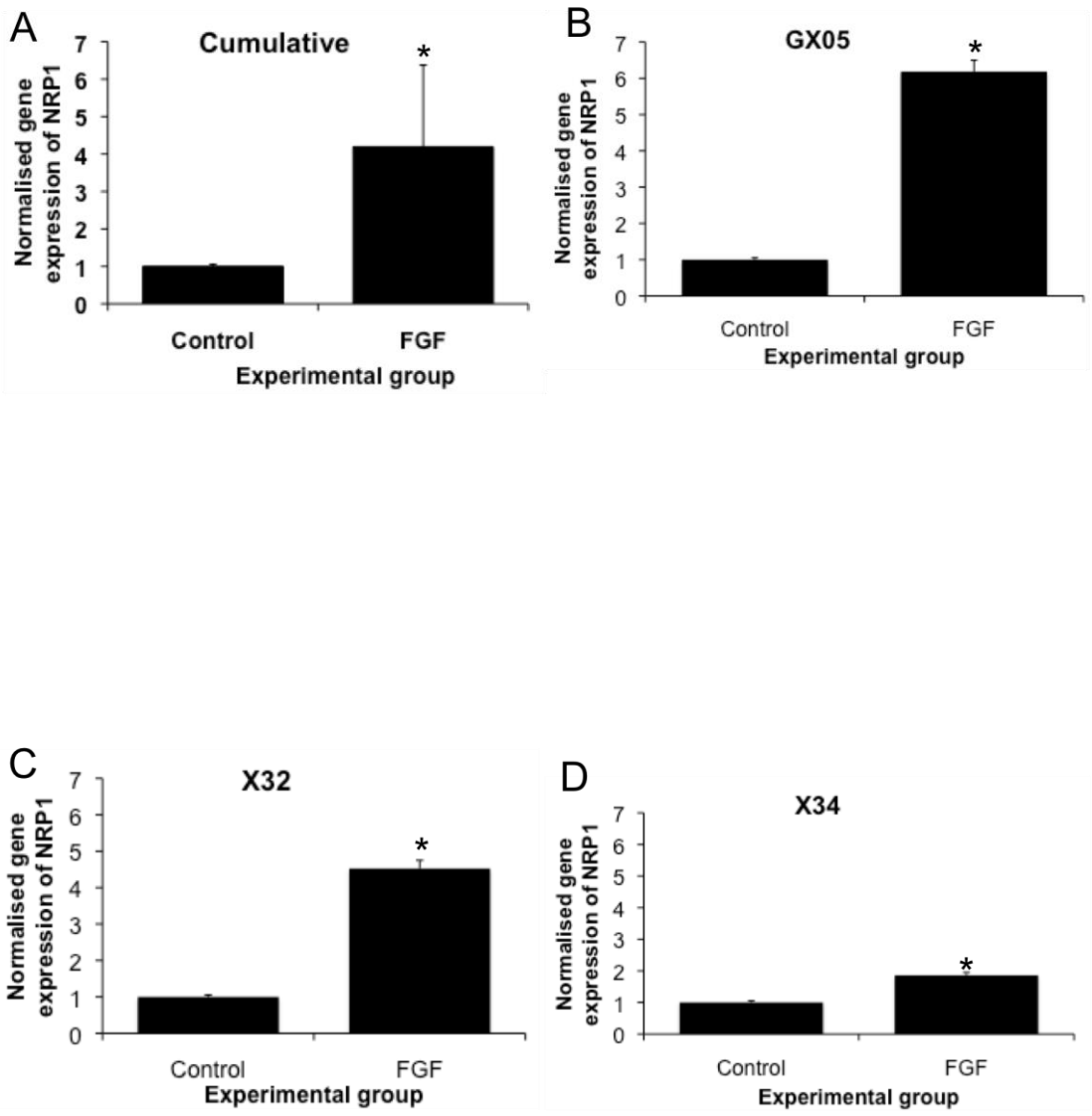



Figure 32 - NRP1 is upregulate in MSCs by bFGF

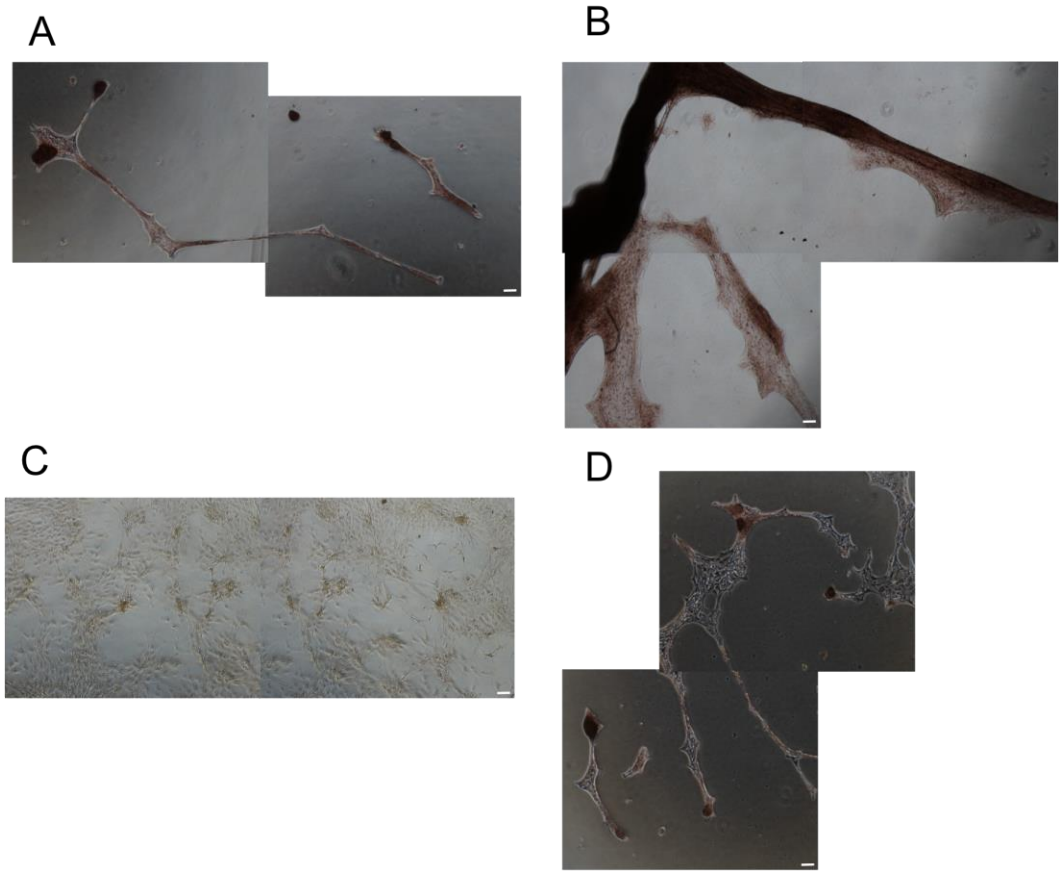
MSCs were stimulated with 10ng/ml bFGF for 12 hours. Cells were harvested and the mRNA isolated. Results presented are cumulative data (A) from three independent donors (B-D). Values =Gene expression for NRP1 analysed by qPCR and normalised to GAPDH & control group ± SEM (n=3) *=$p<0.05$

**Stimulation with bFGF has no effect on tri-lineage differentiation**

Tri-lineage differentiation, post bFGF stimulation, was observed in classical differentiation assays and gene expression studies. In parallel experiments, the impact of NRP1 was also observed with NRP1 KD MSCs.

The osteogenesis protocol showed no significant difference by stimulation with bFGF or knockdown of NRP1, both phenotypically (Figure 33A-D) and gene expression for an osteogenic gene, ALP (Figure 33E).

Stimulation with bFGF had no effect on number of cells with oil red O stained clusters (Figure 34A-D) and PPARg expression, an adipogenic gene (Figure 34 E). However, significantly less MSCs knocked down for NRP1 were oil red O positive than control cells (Figure 34A-D). Knockdown of NRP1 did not effect the expression of PPARg (Figure 34F). All experimental groups displayed similar phenotype in the chrondrogenesis classical assay, staining blue with Alcian Blue (Figure 34A-D).


A
B
C
D

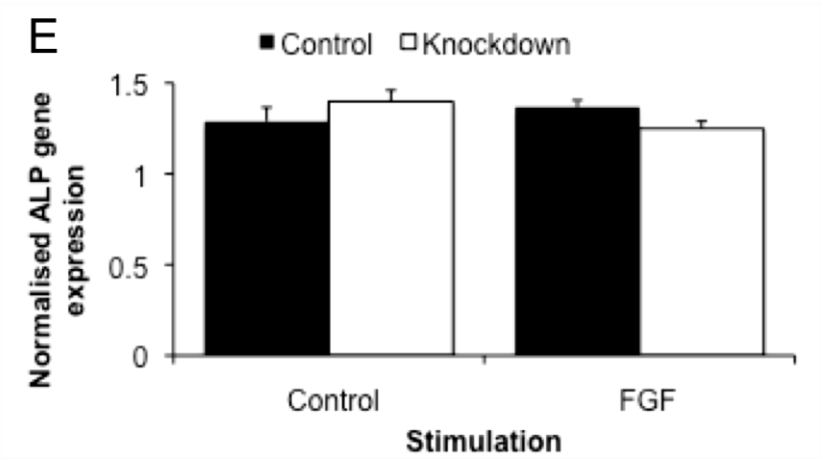
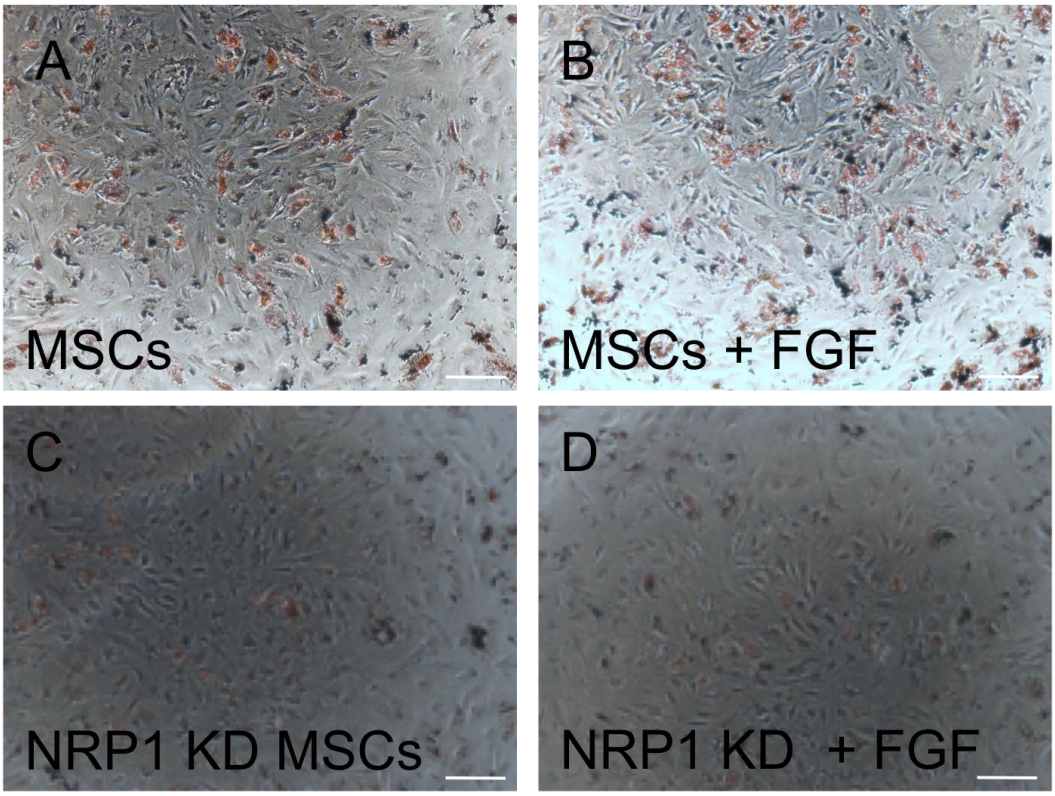



Figure 33 - NRP1 or FGF stimulation have no observed effect on osteogenic capacity. MSCs in all experimental groups were incubated in osteogenic inducing media. After 14 days MSCs were fixed and stained with alizarin red S. Images were taken at x4 magnification and scale bars represent 100µm (A-D). After 24 hours, cells were harvested and mRNA was isolated. Values = Average normalised expression for ALP analysed by qPCR and normalised to GAPDH & control group ± SEM (E) (n=3) *=$p<0.05$


A
MSCs
B
MSCs + FGF
C
NRP1 KD MSCs
D
NRP1 KD + FGF

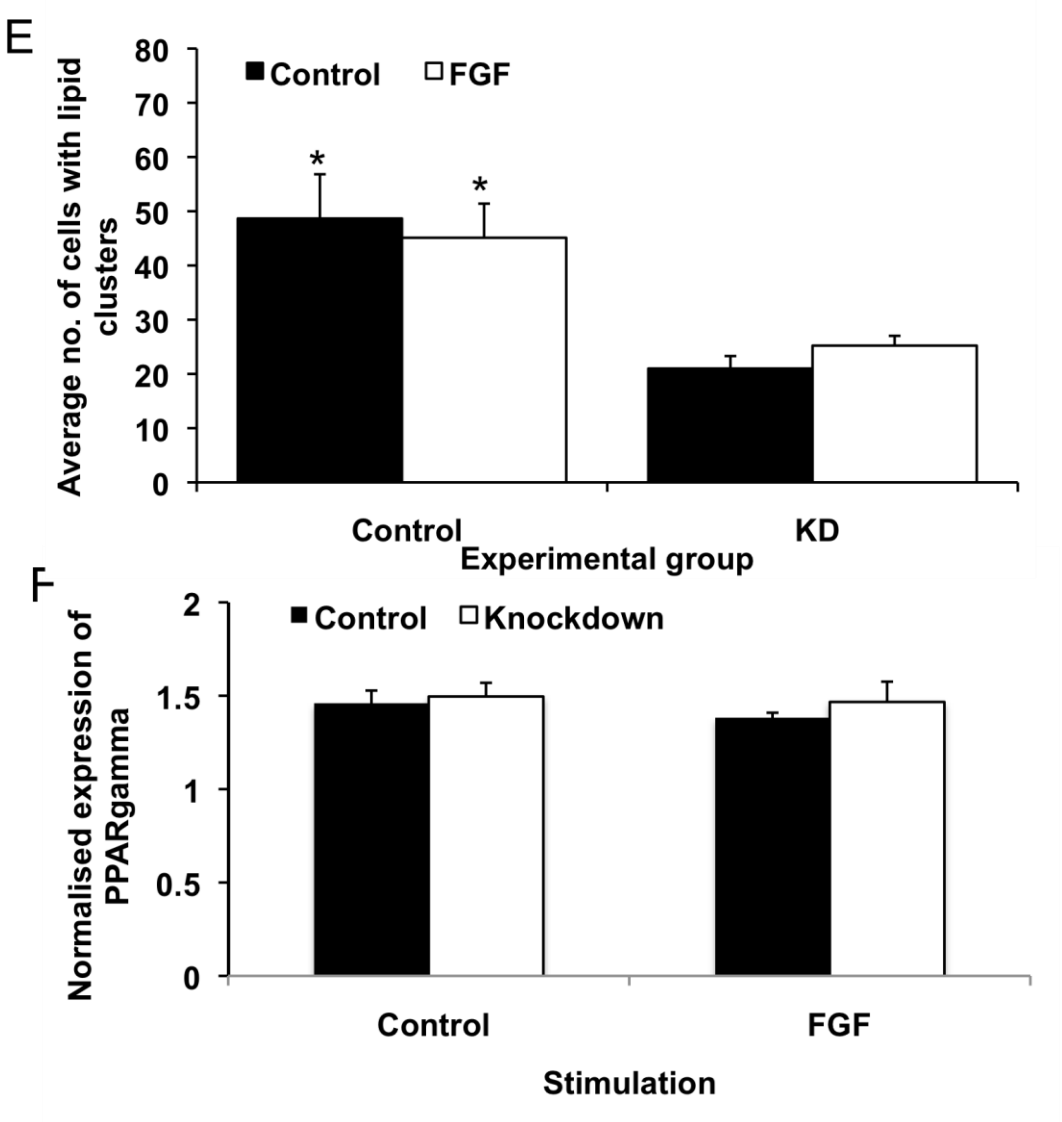



Figure 34 - Knockdown of NRP1 decreases adipogenic capacity

48 hours after transfection with either Nrp1 siRNA or scramble siRNA, MSCs were stimulated with bFGF or unstimulated (control) for 12 hours and incubated with adipogenic inducing media. Cells were fixed after 14 days and stained with 1% oil red O solution. All images were taken at x4 magnification. Scale bars represent 100µm (A-D). Results were the mean + SEM of average number of cells per field view containing lipid clusters ± SEM (n=3) (E). After 24 hours, mRNA was isolated. Values = Average normalised expression for PPARɣ analysed by qPCR and normalised to GAPDH & control group ± SEM (F) (n=3) *=p<0.05)

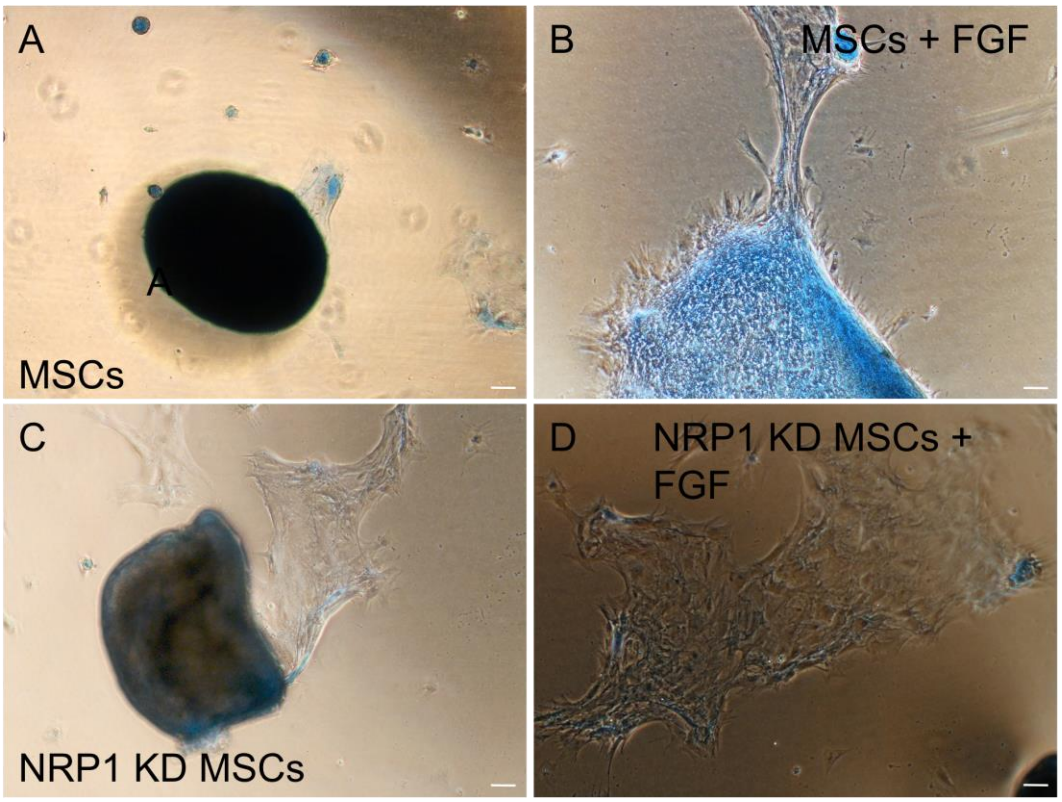


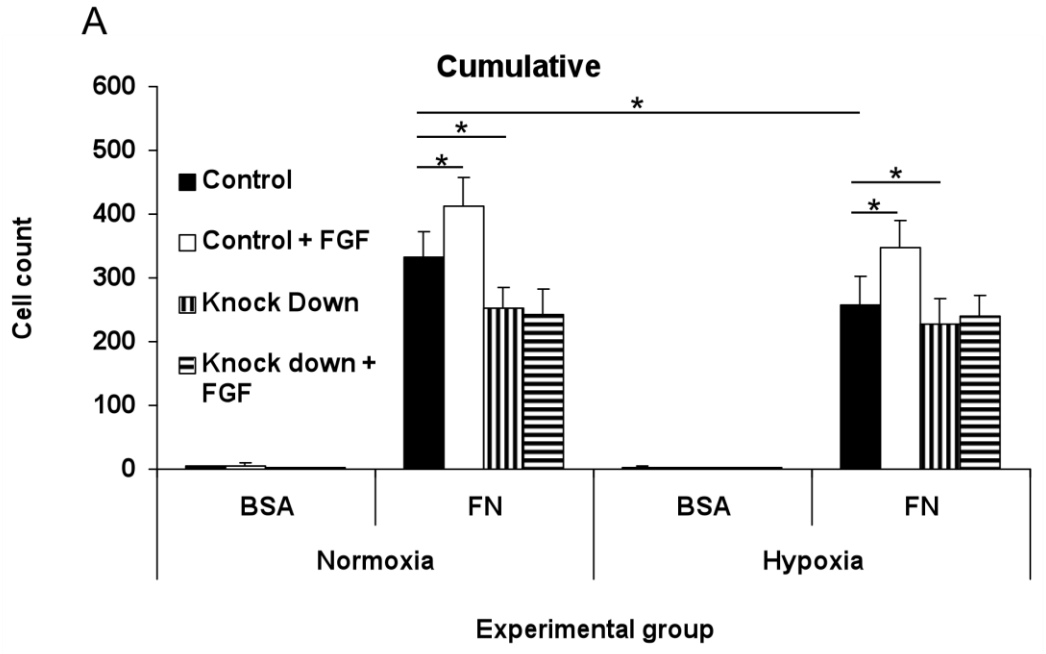

Figure 35 – NRP or bFGF have no observed effect on chondrogenic capacity

48 hours after transfection with either Nrp1 siRNA or scramble siRNA, MSCs were stimulated with bFGF or unstimulated (control) for 12 hours and incubated incubated with chondrogenic media for 14 days. Cells were then fixed and stained with Alcian blue. Images were taken at x4 magnification. Scale bars are equal to 100µm (A-D)

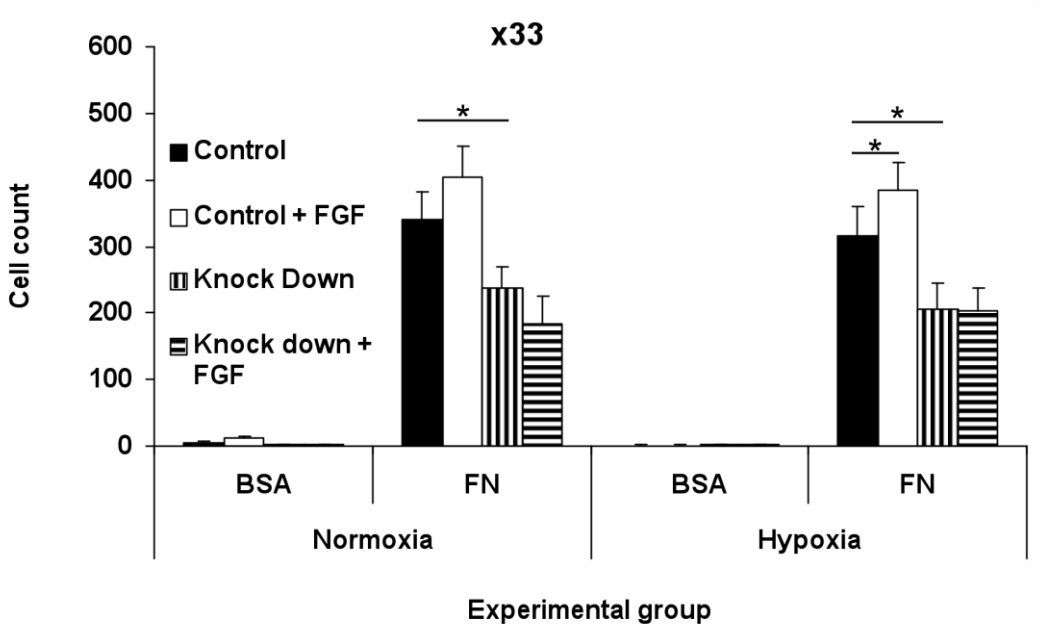
**Adhesion to fibronectin decreased in hypoxic conditions, but is rescued by stimulation with bFGF**

To mimic the environment of the ischemic heart, an in-vitro assay using fibronectin as an ECM substrate and different oxygen tensions (normoxia & hypoxia) were used for an MSC adhesion assay. Cells were seeded

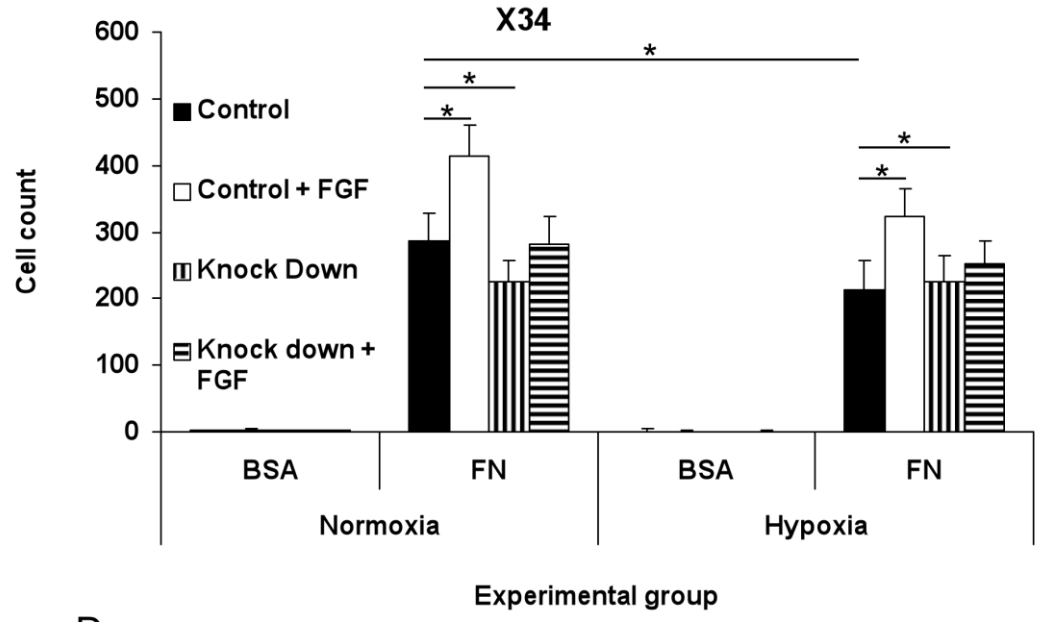
onto fibronectin, or a control substrate of tissue culture coated with BSA. In ambient oxygen levels, bFGF increased cell attachment (p=0.001; figure 36A) NRP1 KD MSCs underwent decreased attachment to fibronectin, and stimulation with bFGF was not able to rescue this effect (Figure 36A). In hypoxic conditions, cell attachment was decreased but this effect was rescued with bFGF preconditioning to that of control levels in ambient oxygen (Figure 36A). This trend was reproduced in all donor isolations used except for X33, which had no significant reduction adhesion to fibronectin in hypoxia (Figure 36B). These results highlight the importance of NRP1 to bFGF stimulated adhesion of MSCs to fibronectin



B


x33
600
500
400
300
200
100
0
Cell count
Control
Control + FGF
Knock Down
Knock down + FGF
*
*
*
BSA
FN
BSA
FN
Normoxia
Hypoxia
Experimental group

C


X34
600
500
400
300
200
100
0
Cell count
Control
Control + FGF
Knock Down
Knock down + FGF
*
*
*
*
*
*
BSA
FN
BSA
FN
Normoxia
Hypoxia
Experimental group

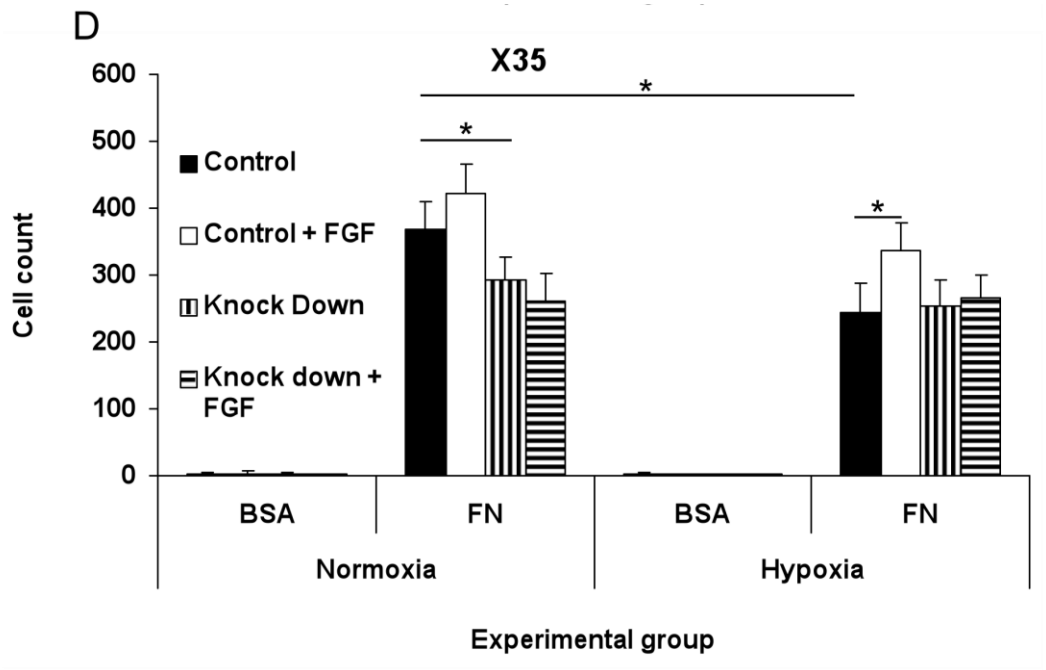


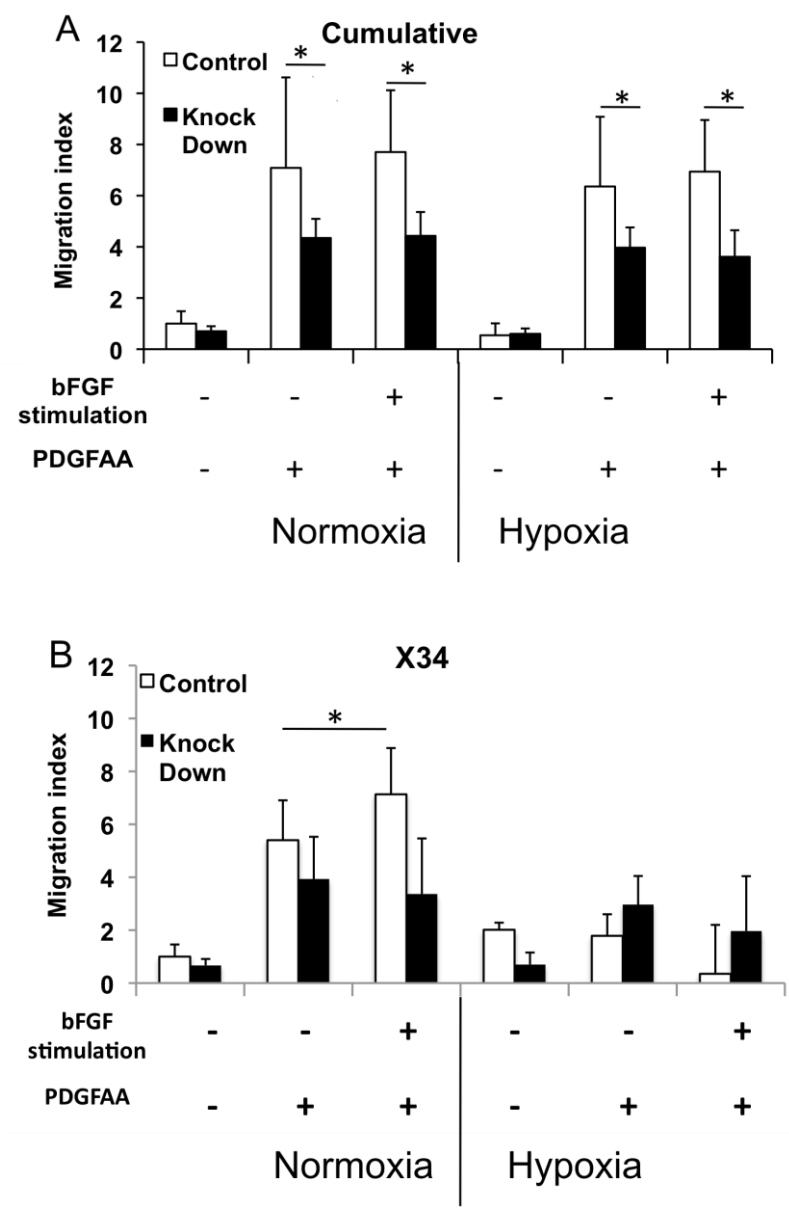

Figure 36 – bFGF increases adhesion of MSCs in hypoxia

48 hours after transfection with either Nrp1 siRNA or scramble siRNA, MSCs were stimulated with bFGF or unstimulated (control) for 12 hours and seeded BSA or fibronectin (FN) coated wells. Results presented are cumulative data (A) from three independent donors (B-D). Values = mean number of cells per field view ± SEM (n=3), *=p<0.05

**Migration is not affected by oxygen tension or bFGF stimulation**

Oxygen tension had no significant effect on the migration of MSCs (Figure 37A). The MSCs, control or stimulated, were seeded into transwells and exposed to PDGF-AA. The chemotaxis of MSCs to PDGF-AA had shown to be NRP1 dependant. As shown previously, migration was decreased when NRP1 was silenced. However, no additional effect was observed by

prestimulating the MSCs. Also, there was no difference in number of cells migrated in 20% versus 2% oxygen.



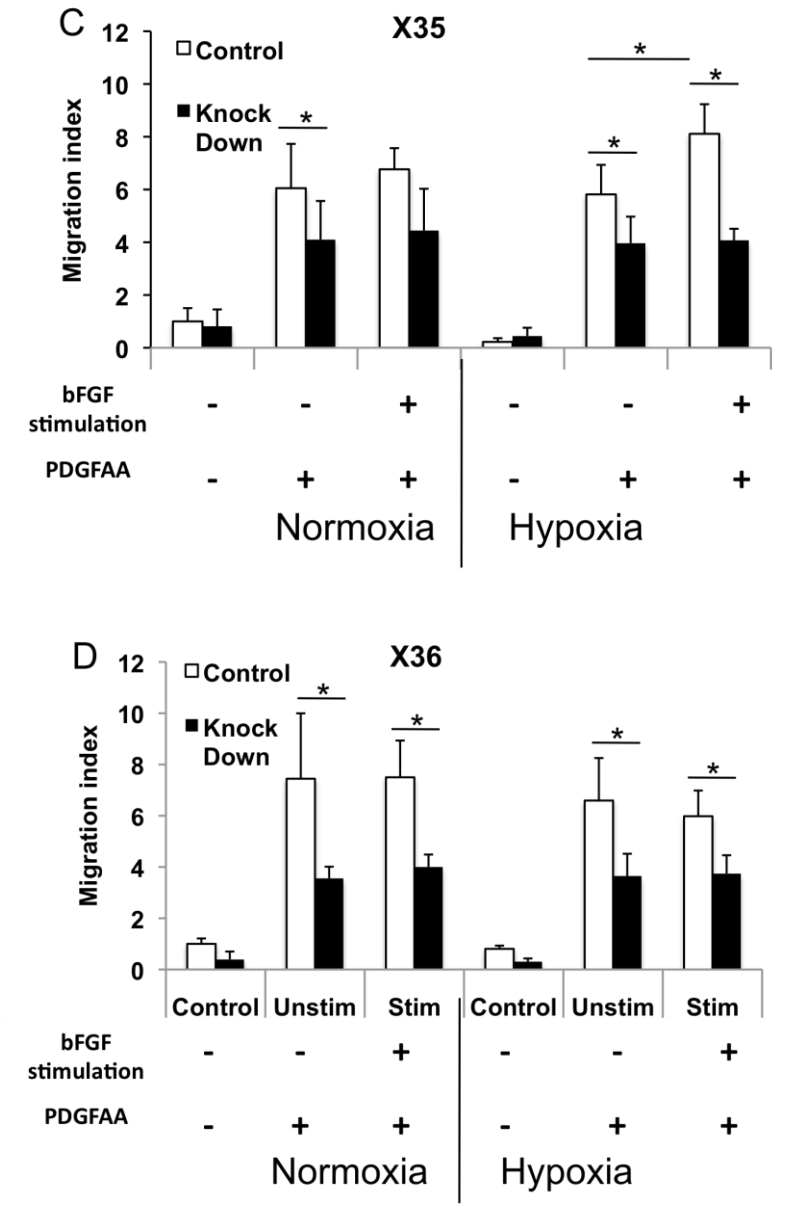



Figure 37 Hypoxia or FGF does not effect MSC chemotaxis to PDGF-AA. MSCs were transferred to transwells and exposed to 50ng/ml PDGF-AA for 3 hours. Non-migratory cells were scrapped off and migrated cells on the membrane were stained with crystal violet. Images were taken at x10 magnification in 3 random field views and counted using ImageJ software. Results presented are cumulative data (A) from three independent donors (B-D). Values = mean migration index ± SEM (n=3), *=$p<0.05$

**MSC proliferation rate not affected by bFGF stimulation**

The effects of NRP1 knockdown on MSC proliferation was investigated in bFGF stimulated and control experimental groups. After stimulation with bFGF, media was replaced with 1% serum growth media and incubated for 24 hours. No significant increase with bFGF-stimulated groups was observed (Figure 38A). This trend was consistent for all isolations (Figure 38B-D) except for X35 that had higher average absorbance value for knockdown MSCs (Figure 38C)

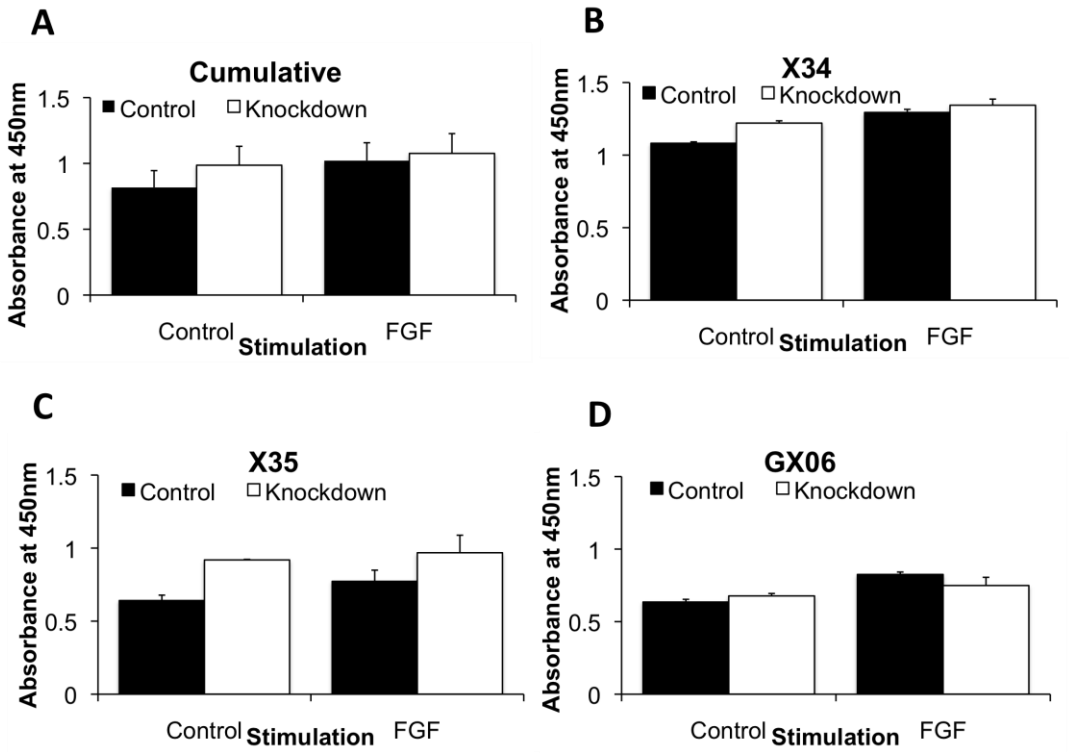



Figure 38 – Stimulation with bFGF does not significantly affect proliferation of MSCs. Following SiRNA knockdown with either Scr control or target NRP1 SiRNA, the effect of bFGF stimulation was analysed after 24 hours. CCK-8 was added to supernatant and measured using a plate reader at

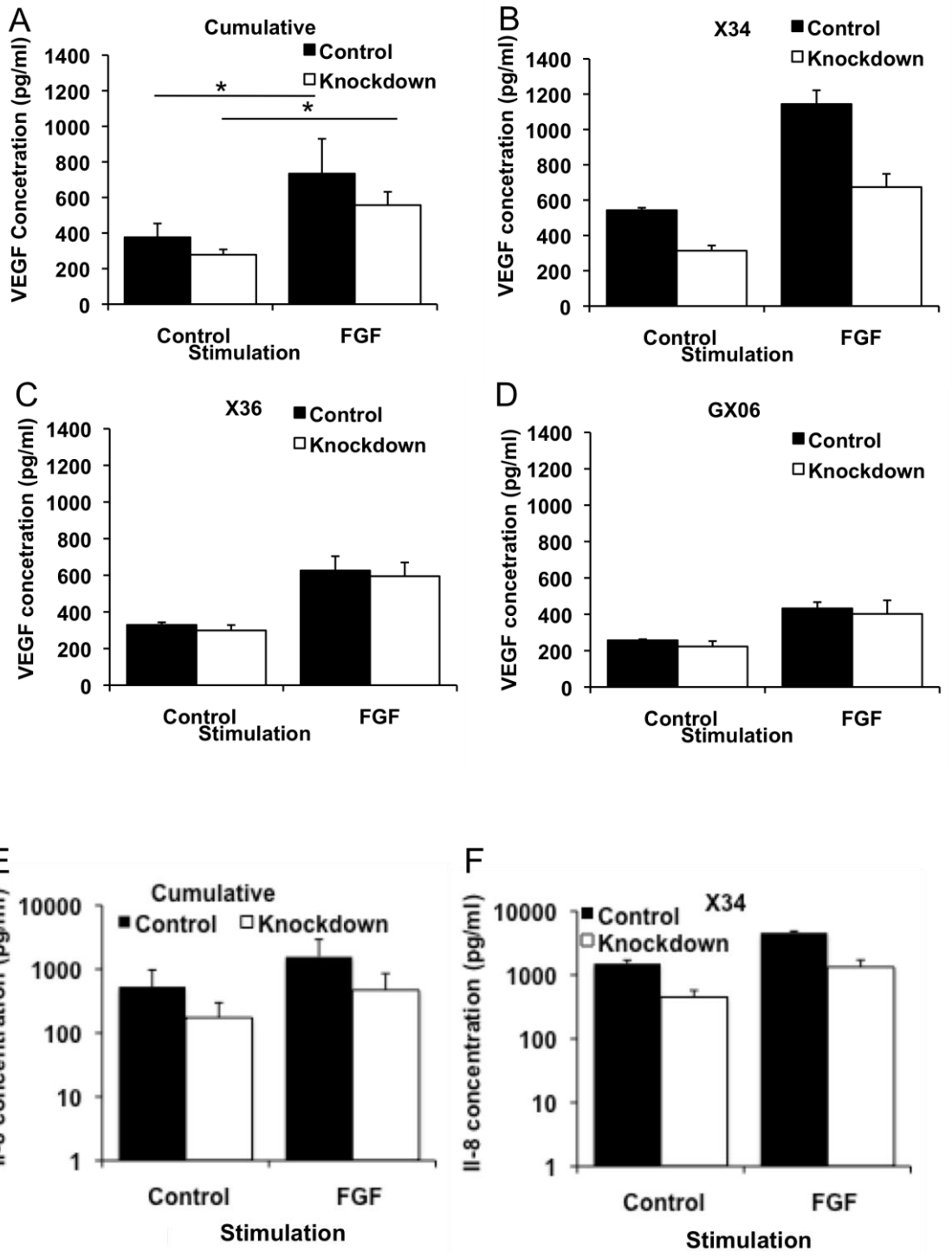
450nm. Results presented are cumulative data (A) from three independent donors (B-D). Values = mean absorbance ± SEM (n=3),

**Stimulation increases the MSC cytokine release profile**

MSCs are hypothesised to release cytokines, such as VEGF, HGF, PDGF-AA and IL-8 that activate pro-angiogenic, survival pathways and account for their immunomodulatroy capacity. The MSC protein release profile was investigated using ELISA after 24 hours for each experimental group. VEGF was significantly increased in the groups that were stimulated ($p<0.01$); however as there was no difference between control MSCs and NRP1 KD groups, VEGF release was not NRP1 dependant (Figure 39A). This trend was consistent in all isolations although stimulation had a greater VEGF release in X34 (Figure39B).

IL-8 was significantly increased in all isolations except in X35 that had lower values of Il-8 that the standardised range. Isolation X34 had higher values of Il-8 than the standardised range (Figure 39G).

No effect in HGF release was observed in experimental groups (Figure 39I). Isolation GX05 had a lower release of HGF than the other 2 isolations (Figure39J). No PDGF-AA was detected from any MSC isolation (no data shown).


A
Cumulative
Control
Knockdown
VEGF Concetration (pg/ml)
1400
1200
1000
800
600
400
200
0
*
*
Control
FGF
Stimulation
B
X34
Control
Knockdown
VEGF concetration (pg/ml)
Control
FGF
Stimulation
C
X36
Control
Knockdown
VEGF concetration (pg/ml)
Control
FGF
Stimulation
D
GX06
Control
Knockdown
VEGF concetration (pg/ml)
Control
FGF
Stimulation
E
Cumulative
Control
Knockdown
Il-8 concentration (pg/ml)
10000
1000
100
10
1
Control
FGF
Stimulation
F
X34
Control
Knockdown
Il-8 concentration (pg/ml)
10000
1000
100
10
1
Control
FGF
Stimulation

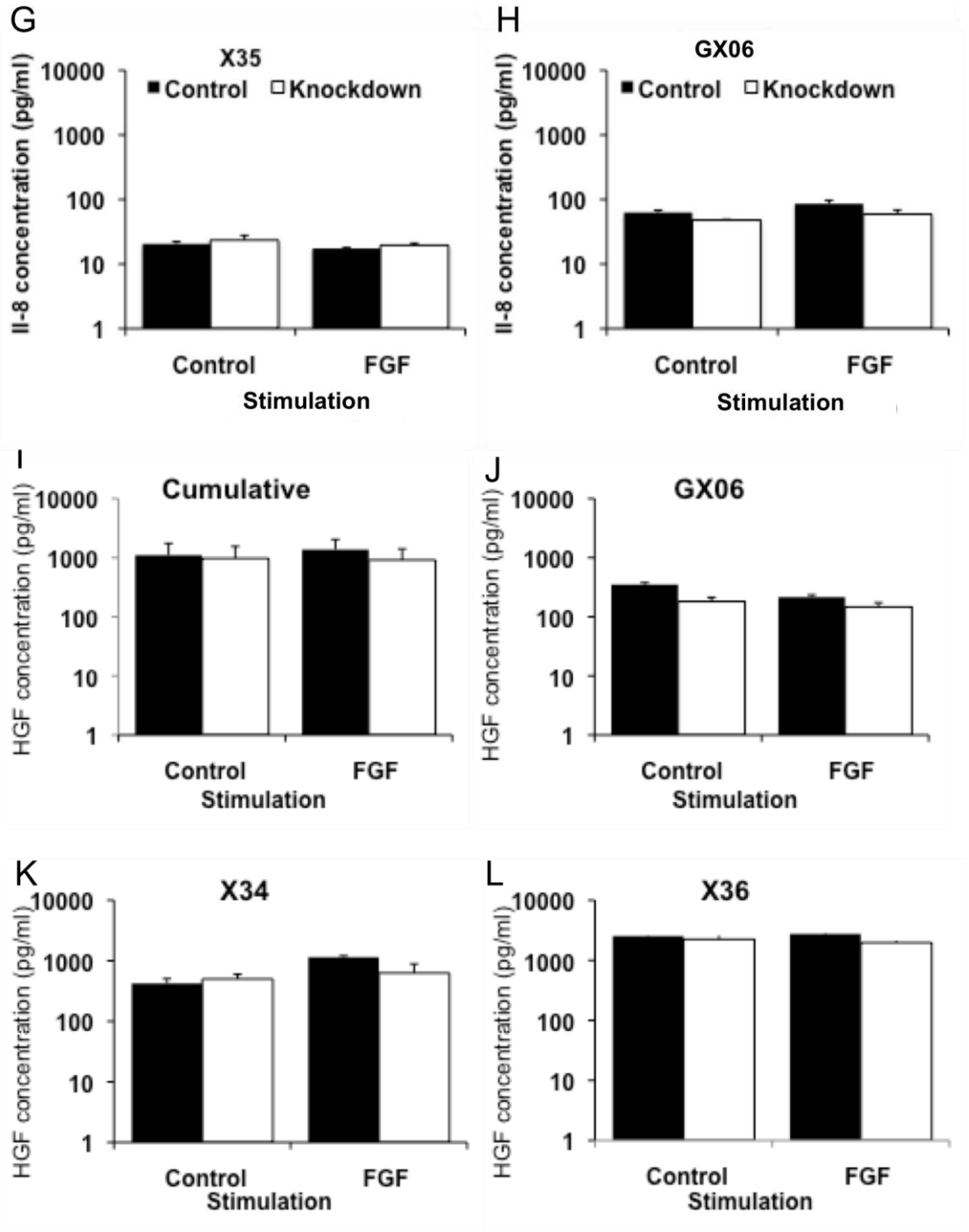



Figure 38 - FGF induces increased VEGF release of MSCs.

MSCs, either control or NRP1 knockdown were exposed to 10ng/ml bFGF for 12 hours, then media was changed to 1% FBS in DMEM. After 24 hours media was removed and analysed for VEGF (A-D), IL-8 (E-H), and HGF (I-L) by ELISA. No PDGF-AA was detected in any isolation. Results

presented are cumulative data (A,E,I) from three independent donors. Values = Average protein concentration ± SEM (n=3), *=p<0.05

## Discussion

The previous chapter identified NRP1 as an important protein for attachment and migration and assays were developed to ascertain the functional characteristics of the cells. Passage 3 MSCs proved to be optimal for achieving suitable cell quantities without diminishing cell function, as culture to passage 5 proved to reduce the migratory capacity of the MSCs. The assays developed aim to more closely replicate the physiological environments of the infarct heart.

Preconditioning MSCs with molecules that might stimulate or enhance their activity is an exciting candidate bioprocessing strategy to prime MSCs and achieve optimal product quality. Improving both attachment and pro-vasculogenic capabilities can lead to a synergistic effect in the sense that the more cells that are retained in the region of interest with a higher potency, the greater the therapeutic response. This would be of benefit to a bioprocess as lower therapeutic dose can be used.

Although genetically enhancing stem cells has been very successful in pre-clinical trials there is still much safety & technical concerns associated with this approach (Bonaros et al., 2008; Penn & Mangi, 2008). An ex-vivo pharmacological preconditioning using factors that are already licensed for use in humans is a more attractive strategy. This study investigated the

effect of upregulation of NRP1 on adhesional, migratory and cytokine responses.

This is the first study to investigate the effect of upregulation of NRP1 on the biological function of MSCs. NRP1 expression and regulation has not been well characterised in MSCs, but it is known to be vital for angiogenesis in endothelial cells (Valdembri et al., 2009). Also, in cancer it is involved in tumour growth and invasion (Stephenson et al., 2002) as NRP1 over expression in prostate carcinoma is correlated with metastatic potential and tumour aggressiveness (Latil et al., 2000).

NRP1 was increased at both gene and protein level after stimulation with bFGF, while hypoxia caused NRP1 expression to decrease in both acute and longer term, 24 hour, time periods. In addition to upregulating NRP1, bFGF has shown to activate the PI3K/Akt signalling pathway that has a core regulatory role in MSC survival, proliferation, migration and differentiation (Ahn et al., 2009 {Chen, 2013 #470; Chen et al., 2013). Therefore, preconditioning with bFGF was used for the rest of the study.

To validate the MSCs as multipotent, tri-lineage differentiation protocols were performed on knockdown and preconditioned MSCs. To which all demonstrated tri-lineage differentiation to some extent. Increase in osteogenic potency has been previously observed when in long term culture using high concentration of bFGF, whereas the short term stimulation in this study had no effect either phenotypically or on ALP gene expression (Hanada et al., 1997). However, knockdown of NRP1 significantly reduced the number of oil red O positive lipid clusters that are

an indicator of adipocyte like cells. As no change was observed in PPARg, an early adipogenic marker, NRP1 is more likely to influence the maturity of preadipocytes to lipid containing adipocytes (Cristancho and Lazar, 2011). This role of NRP1 for adipogenic differentiation has yet to be fully investigated.

bFGF preconditioning significantly improved attachment of MSCs in both normoxia and hypoxia. Significantly fewer MSCs attached to fibronectin in a hypoxic environment than normoxia. This response was rescued by preconditioning the cells with bFGF. As stimulation of NRP1 knockdown MSCs did not result in enhanced adhesion as occurred for control MSCs this is likely to be an NRP1 dependent mechanism. The hypoxic conditions mimicked physiological sites, therefore improving adhesion in these conditions would be of great benefit for a therapeutic candidate for cell therapy. As many transplanted cells do not survive or engraft, enhancement of this response could improve clinical end outcomes (Kean et al., 2013).

In vascular smooth muscle cells, upregulation of NRP1 resulted in enhanced migration (Liu et al., 2005). However, in this study no further increase in migration to PDGF-AA was observed for MSCs.

Biological function assays are vital for understanding the quality attributes of MSCs products for a therapeutic indication. The results show that in different isolations, from healthy donors, there is a large difference in cytokine release. MSCs have been proposed to act as 'Medicinal Signalling Cells' (Koutsoumbelis and Grande, 2013), and therefore, on this

premise an ideal MSC candidate for therapy could be decided on the basis of secreted factors. One such factor is VEGF which is cited in many studies for it's pro-angiogenic effect (Gnecchi et al., 2008b; Wang et al., 2006). On average, bFGF stimulated MSCs produced approximately 2 fold more VEGF than unstimulated cells. This was independent of NRP1, as the same effect was observed in the knockdown MSCs

Studies have shown that in addition to VEGF, HGF is cardio-protective and can maximise beneficial effect of MSC following cardiac injury (Deuse et al., 2009). However, HGF release was not affected by stimulation with bFGF. This is contrary to another study, using adipose derived stromal cells, which showed a substantial increase in HGF after culturing with 10ng/ml bFGF (Suga et al., 2009).

Another use of MSCs has been for their immunomodulatory properties in indications such as graft versus host disease. For this a high release of IL-8 such as X34 would be highly desired. There was a substantial difference between the release profiles of individual isolations, with an approximate 100-fold difference between X35 and X34. Proliferation was not affected by preconditioning during the same time period for which the ELISA protocol was performed. Therefore, change in proliferation did not account for any change in growth factor release. This response to bFGF stimulation is consistent with another study that shows a significant increase long term, +6 days, but not in shorter time periods (Suga et al., 2009)

## Conclusion

In conclusion, this study showed that *ex vivo* preconditioning MSCs with bFGF increases adhesion of cells in hypoxia in a NRP1 dependent mechanism. Preconditioning also enhanced release of VEGF, a potent angiogenic growth factor, independent of NRP1. A non-genetic approach of enhancing the MSCs performance could have a synergistic positive effect for use in therapy. If more, higher potency cells can engraftment after therapeutic application, then fewer cells will be needed to achieve the same functional output. This is highly advantageous for cell therapy bioprocessing.

# Chapter 6 - Discussion

Heart failure is a global problem with no cure, contributing to considerable costs to the economy. Although improvements in pharmaceutical and surgical interventions have increased patients' survival after myocardial infarction, the number of patients progressing to congestive heart failure has increased for which there is no cure, only symptomatic control, has increased (Pearson-Stuttard et al., 2012). Cell therapies are an emerging class of therapeutics that have the potential to address current unmet clinical needs including heart failure. Human bone marrow MNCs and MSCs are both candidates for cellular therapy that are in late phase clinical trials around the world. However, results from these have, so far, been modest (Clifford et al., 2013). Animal studies have shown that cellular survival and engraftment at the site of delivery is low and this possibly explains why clinical improvements are limited (Freyman et al., 2006a). Developing assays that more closely reflect the *in vivo* environment and cell activation strategies that improve the retention of cells should enable better understanding mechanism of action and potency in addition to assay development to accurately measure product quality. Then the clinical potential of these therapies can be more widely achieved.

The proposed function of bone marrow derived stem cells in the ischemic myocardium is widely debated (Mirotsou et al., 2011). However, an attractive

proposed mode of action relates to the cells' vascular support capacity, due to the ischemic nature of the injury sites. The purpose of this thesis was to employ *in vitro* assays that mimicked the physiological environment of the peri-infarct regions to quantify adhesion, migration and pro-vasculogenic properties of the cells. These assays could then be used to assess the effect of preconditioning cells with various biochemical or physiochemical factors as a tool for enhancing these critical functions. Both bone marrow derived MNCs and MSCs were used in this study. The MNCs were obtained from patients in the REGENERATE clinical trial, while the MSCs were cultured from using cryogenically preserved frozen bone marrow aspirates from healthy donors.

The initial hypothesis tested was that by preconditioning the MSCs and MNCs with SDF-1 would enhance the above biological function of the cells by increasing cell attachment and migration responses, based on the fact that SDF-1 is a chemoattractant that mobilises MSCs via CXCR4, which in turn is known to activate integrins (Peled et al., 2000). The other critical reason for examining SDF-1 was based on reported observations from Pasha et al who found that in response to SDF-1 rat MSC retention in an experimental infarct model was increase resulting in improved vascular restoration and decreased infarct size (Pasha et al., 2008). Unfortunately, the SDF-1 preconditioning strategy that was successful for rat AMI using rat MSCs was not reproduced in assays developed in this thesis using human MSCs and MNCs. This may be due to diminished expression of CXCR4, the corresponding receptor of SDF1, as long-term expansion culture has been shown to abrogate CXCR4

expression in hMSCs (Jones et al., 2013; Wynn et al., 2004). No effect was observed after SDF-1 preconditioning for either cell type. Both MSCs and MNCs adhered strongly to fibronectin, but this was substantially diminished in low oxygen tensions. The MSCs did demonstrate vasculogenic potential by forming tubule-like structures when cultured on Matrigel without differentiating into endothelial cells.

Using a hypoxia chamber I was able to reproduce low oxygen conditions (2% oxygen) to reflect that of the physiologic sites that the cells would be transplanted into. In these reduced oxygen conditions adhesion of MSCs and MNCs was significantly impaired when compared to ambient oxygen levels typical of standard culture conditions used for cell bioprocessing. This has significant implications for clinical therapy because processing and quality attributes and survival capacity on delivery to a physiologic inury site and may explain why so few residual cells are retained after dcelivery in animal AMI models, as most process conditions do not reflect the variable physiological conditions characteristic of injury sites. With reduced engraftment in the region of interest there is less opportunity to infer therapeutic benefit, via trophic factors or direct interaction with vascular networks and cardiomyocytes.

The lack of availability of the MNCs from the REGENERATE-IHD clinical trial discontinued their use in the rest of the thesis. Regardless, there is less scope for a bioprocess step for MNCs, as most preconditioning protocols are for long term stimulation that would significantly impair the functional viability

of the cells. MSCs provide a more scalable approach, amenable to cell bioprocessing, as the cells can be expanded to desired quantities and cell characteristics such as surface markers, migration potential or cytokine production can be easily measured. The MSCs used were cryogenically preserved for a repeated experiments and future use. This is consistent with other studies reporting that long-term cryopreservation of human MNCs does not impair the MSC population leading to successful culture (Shen et al., 2012) that proliferates and undergoes tri-lineage differentiation (Ginis et al., 2012).

In the absence of observable difference in cell adhesion responses due to SDF-1, we turned our attention to a second candidate molecule for control of cell adhesion and vascular support, Neuropilin 1. NRP1 is a VEGF co-receptor that is thought to be critical for endothelial cell attachment and migration on fibronectin and angiogenesis (Valdembri et al., 2009) and vascular smooth muscle cell migration to PDGF-AA (Pellet-Many et al., 2011). NRP1 is also expressed on MSCs (Ball et al., 2010) yet it's precise functions are still to be determined. Therefore we hypothesized that NRP1 would have a key role in cell adhesion responses and vascular support phenotype of MSCs.

The assays developed and optimised during the first chapter were used to deterine the requirement of NRP1 in attachment, migration and vascular support MSCs. By selectively knocking down NRP1 with siRNA the functional requirements of the protein could be analysed. NRP1 was important for

attachment of MSCs to fibronectin, but not other extracellular matrix proteins, however it's knockdown did not completely block cell attachment, indicating that other NRP1-independent pathways can partially rescue the defect. In addition, a significant decrease in chemotactic cell migration towards PDGF-AA was seen and then in terms of a potential vascular support role of MSCs, knockdown of NRP1 impaired their ability to form tubule-like strucures and support a pre-exisiting endothelial network.

The final chapter investigated the functional effect of upregulating NRP1. Previous reports have suggested that regulation of NRP1 is dependent on cell type with the same strategies resulting in polarizing effects. For example, some researchers found that NRP1 was increased by hypoxia in a HIF1 dependant fashion (Ellis, 2006), whereas others found that hypoxia decreased NRP1 (Jogi et al., 2004). MSC studies in this thesis demonstrate that gene expression of NRP1 is down regulated following overnight culture in low oxygen conditions. Overnight stimulation with bFGF was shown to increase the expression of NRP1, in a proliferation-independent fashion. Stimulation with bFGF increased MSC adhesion to fibronectin in a NRP1 dependent mechanism, but did not significantly impact on migration to PDGF-AA. Preconditioning with bFGF increased the VEGF expression that has pro-angiogenic effects. Results from this research could in future work move to *in vivo* models to identify if the in vitro improvements observed translate to enhanced therapeutic outcomes.

## *1.1 Implications for MSCs as a therapeutic candidate*

The development of cell therapy is unlike that of a pharmaceutical compound, for which the mechanism of action is known and can be robustly quantified. MSCs products have been developed by several companies are candidates in several companies targeting multiple indications including graft versus host disease and myocardial infarction. However, the precise mechanism is not fully characterised and potency assays for release are not yet standardized or widely available. This lack of knowledge is a current bottleneck to improving therapeutic outcome by means of bioprocess optimisation. The results from this study show that MSCs release a multitude of growth factors that have pro-angiogenic, cytoprotective and immunomodulatory properties, such as VEGF, IL-8, and HGF. If MSCs are preconditioned to have enhanced retention (a function of attachment and migration) at the psychological site of interest, alongside an improved cytokine release profile, this would synergistically improve the therapeutic outcome.

Pharmacological preconditioning of MSC before therapeutic use is an effective strategy as a prerequisite to enhance MSC function. Other strategies include genetic manipulation to over express certain adhesion molecules, or secrete particular proteins. Preconditioning with bFGF can be incorporated as part of the manufacturing bioprocess and then removal of bFGF from the final product assessed as part of the post-processing quality control assessment

This study found that MSCs can interact directly with, and support a pre-existing endothelial network. This is vital requirement post-infarction in a site in which not only are cardiomyocytes are lost but also the vascular network. In addition to indirect support, by paracrine factors such as VEGF, direct interactions would possibly facilitate support and regerenation of the diminished network by acting as a stromal support cell to the at risk vessels.

There is much contention over the definitive identity of a MSC. At the start of the thesis the International Society of Cell Therapy (ISCT) defined a MSC as plastic adherent; CD105, CD73, and CD90 positive CD45, CD14 and CD19 negative; with the ability to differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al., 2006). However, other studies have demonstrated similar characteristics in perivascular and fibroblast cells (Covas et al., 2008a). Importantly, *in vitro* and *in vivo* models are unequivocal for clinical purposes. The assays developed in this thesis, particularly the endothelial support assay, offer a potential to correlate biological function in *in vitro* assays that more closely reflect physiologic environments. A new working proposal by the ISCT concentrates on the immunogenicity of MSCs such as response to IFN and TNFα, and indolamine 2,3 dioxygenase (IDO) release (Krampera et al., 2013). Defining a MSCs in this manner would not be suitable for an indication such as acute myocardial infarction where the proposed mechanism of action is vasculogenic and cytoprotective. During the entire study, MSCs were only characterized according to the original 2006 ISCT criteria for continuity.

This study adds more evidence that MSCs are also similar to pericytes in function. The live cell staining in the network support assay clearly shows the MSCs bridging/communicating between the endothelial cells in a pericyte like fashion that was diminished when NRP1 was knocked down. Blocki et al, indicate that this may only represent a subpopulation of MSCs having a specialised role in vascular biology (Blocki et al., 2013)

## *1.2 Heterogeneity of MSC isolations*

Throughout the thesis there was variability between the different MSC isolations, despite adoption of consistent isolation protocol and culture conditions. This was particularly evident in the protein release ELISAs whereby one donor isolation produced extremely high VEGF levels, up to three fold greater than that of other isolations. However higher release of one protein did not correspond to overall high protein release. Large donor variability accounted for differing response to bFGF stimulation. This is relevant to commercial scale manufacturing, because for an allogenic product an optimal MSC isolation should be chosen with the critical quality attributes indicated to achieve a greater clinical outcome. An isolation with increased engraftment and release of pro-vasculogenic cytokines, like VEGF, would be more suitable as a therapeutic candidate. On the other hand for autologous therapy, identifying patients whose cells do not produce a defined minimum threshold of growth factor levels or functional outputs would potentially provide a predictive measure of clinical failure and hence help to stratify the patient populations and improve the clinical success of cell therapies

Donor variation has been documented in several studies and Siegel et al identified HGF secretion as negatively correlated with expression of CD71, and CD140b (Siegel et al., 2013). Another study involving 17 donor isolations identified differences in growth rate and osteogenic potential (Phinney et al., 1999). Inter-donor heterogeneity can be attributed to donor age and sex (Zhou et al., 2008).

## *1.3 Conclusion*

Bone marrow derived stem cells are a suitable candidate for therapeutic use post AMI. This study developed suitable functional *in vitro* assays as a model to characterise these cells and investigate strategies to improve their retention and pro-vasculogenic capacity. The initial hypothesis that SDF-1 would enhance these functions in MSCs and MNCs was unfounded, so an investigation into adhesion proteins, specifically NRP1, was investigated to ascertain it's role. NRP1 was shown have an important role in adhesion and pro-vasulogenic function of MSCs. By upregulating NRP1 with bFGF, adhesion to fibronectin in hypoxic conditions that was significantly diminished was then rescued to that of normal oxygen tensions. These findings have for the potential to be translated for the successful improvement of a bioprocess of a cell therapy candidate for the prevention of heart failure.

## *1.4 Future work*

This investigation highlighted the importance of NRP1 in MSCs, and the potential for preconditioning by bFGF to be used to improve their biological function. The following investigations can take this work further:

1. Development of the assays to validate the function of MSCs and use either as predictive tools to stratify and hence identify the most suitable patient candidates for autologous therapy or as identity/potency assays to provide a measure of key critical quality attributes
2. Elucidation of the importance of biochemical pathways using knockdown or pre-stimualtion strategies
3. In vivo MI model, or ex vivo langendorff, using bFGF preconditioned MSCs

The cell therapy industry lacks appropriate release assays and validation of MSCs with no current standards in place. The assays I developed during this investigation can be further developed, and correlated to either surface marker expression, or protein secretion, which would then provide a surrogate measure of function. For instance, high adhesion may be associated with high integrin activation, or angiogenic response correlated to by VEGF secretion. To date, most release assays for MSCs involve the immunomodulatory function of MSCs that have more relevance for indications like GvHD. Biological release assays need to be developed that are more

relevant to mechanism of repair in AMI. *In vivo in vitro* correlation (IVIVC) is used for the development of small molecule drugs and provides a good predictive model for quality control that allows flexibility for process changes. The large donor-to-donor variability demonstrates how important it is develop potency assays that assess cellular function, not just their expression of arbitary surface markers or differentiation. In this addition, one limitation of this study was that although low oxygen was accounted for in the assays, other physiochemical conditions at injury sites such as pH changes due to oxidative stress were not accounted for. Therefore, implications of factors such as acidosis should be investigated to assess the impact on adhesion and migration.

NRP1 has a multifaceted role being important for adhesion, migration and a vascular support function of MSCs. Identification of the downstream pathways NRP1 is involved with could lead to greater knowledge of mechanism of action of MSCs. The contribution of NRP1 to neovascularisation on other cell types, including endothelial cells, VSMCs and bone marrow mononuclear cells has been well documented, including it's interacting with VEGFRs, PDGFRs, Semaphorin3a, and α5β1 (Fujisawa, 2002; Pellet-Many et al., 2011; Valdembri et al., 2009; Zacchigna et al., 2008). Although, MSCs do not express VEGFRs, VEGF-A is channeled through PDGFRs (Ball et al., 2010). Co-immunopreciptation of NRP1 during adhesion to fibronectin and tubule formation would identity vital interactions.

In addition, although NRP1 knockdown had no effect on scratch wound closure on a fibronectin substrate, future immunoflourescence analysis would identify possible translocation of NRP1 to the leading edge of migration as postulated by Valdembri et al (Valdembri et al., 2009). This study showed the colocalisation of α5β1 integrin in trafficking vesicles and adhesion sites of endothelial cells. Investigation in MSCs using total internal reflection fluorescence (TIRF) would assess this interaction, via fluorescence resonance energy transfer (FRET), between α5β1 integrin and NRP1.

MSCs preconditioned with bFGF demonstrated a novel way of improving engraftment and vascular support function in *in-vitro* assays replicating the infarct physiological site. This can be taken forward into *in vivo* models to identify the translational potential of this strategy. Initial studies would use the chick embryo chorioallantoic membrane (CAM) assay to demonstrate neovascularisation *in vivo* as a relatively rapid protocol to follow on from the tubule network assays developed *in vitro* on matrigel. To estimate attachment a Langendorrf model can be used to analyse the change efflux of cells transplanted over time either intracoronary or intramyocardial after preconditioning. A collaborating lab has shown the majority of MNCs to efflux the Langendorff heart 5 minutes after injection intracoronary (data not published). Further to results from these models, preconditioned MSCs vs control MSCs would then be used *in vivo* using a rat MI model after left

anterior descending (LAD) occlusion. To identify and quantify engrafted MSCs in situ hybridization would be used for the human Y chromosome.

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